Helsinki University Biomedical Dissertations No. 91

DEFECTS IN TRICARBOXYLIC ACID CYCLE ENZYMES FUMARATE HYDRATASE AND SUCCINATE DEHYDROGENASE IN CANCER

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Academic dissertation

To be publicly discussed, with the permission
of the Medical Faculty of the University of Helsinki,
in lecture hall 2, Meilahti hospital, Haartmaninkatu 4,
on May 25th, 2007, at 12 noon.

Helsinki 2007

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ISBN 978-952-10-3970-6 (paperback)
ISBN 978-952-10-3971-3 (PDF)
ISSN 1457-8433
http://ethesis.helsinki.fi/
Yliopistopaino
Helsinki 2007

CONTENTS

ABBREVIATIONS

A	adenine
AI	allelic imbalance
APC	adenomatous polyposis coli
ATP	adenosine triphosphate
BHD	Birt-Hogg-Dubé syndrome
bp	base pair
BRCA1	breast cancer 1
C	cytosine
CDK4	cyclin-dependent kinase 4
CHEK2	checkpoint kinase 2
cDNA	complementary deoxyribonucleic acid
DHPLC	denaturing high performance liquid chromatography
DNA	deoxyribonucleic acid
dNTP	dinucleotide triphosphate
ETC	electron transport chain
FAD/FADH	flavin adenine dinucleotide
FH	fumarate dehydrogenase, fumarase
FHD	fumarate hydratase deficiency syndrome
FHv	fumarate hydratase gene variant
FHIT	fragile histidine triad
FLCN	folliculin
FMM	familial malignant melanoma
G	guanine
GFP	green fluorescent protein
GIST	gastrointestinal stromal tumour
HGF	hepatocyte growth factor
HIF1	Hypoxia-inducible factor 1
HIF1α	Hypoxia-inducible factor 1, alfa subunit
HIF1β	Hypoxia-inducible factor 1, beta subunit
HLRCC	hereditary leiomyomatosis and renal cell cancer
HNPCC	hereditary non-polyposis colorectal cancer
HPRC	hereditary papillary renal carcinoma
HPT-JT	hyperparathyreoidism-jaw tumour syndrome
HRAS	Harvey rat sarcoma viral oncogene homolog
HRPT2	hyperparathyroidism 2
HSP	heat shock protein
KIT	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog
KRAS	Kirsten rat sarcoma viral oncogene homolog
LFS	Li-Fraumeni syndrome
LOH	loss of heterozygosity
MCUL	multiple cutaneous and uterine leiomyomata
MEN2	multiple endocrine neoplasia type 2
MET	met proto-oncogene (hepatocyte growth factor receptor)
MLH1	MutL (*E. coli*) homolog 1
MMR	mismatch repair

mRNA	messenger ribonucleic acid
MSH2, -6	MutS (*E. coli*) homolog 2 and 6
NAD^+/NADH	nicotinamide adenine dinucleotide
NRAS	neuroblastoma rat sarcoma viral oncogene homolog
NUD	*non ultra descriptus,* undefined
OMIM	online mendelian inheritance in man
p	short arm of a chromosome
PCC	pheochromocytoma
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PGL	paraganglioma
PHD	prolyl hydroxylase
PMS	postmeiotic segregation increased
PTEN	phosphatase and tensin homolog
p53	tumour protein 53
q	long arm of a chromosome
RAS	Harvey and Kirsten sarcoma virus gene
RB1	retinoblastoma gene
RCC	renal cell carcinoma
RET	ret proto-oncogene
RNA	ribonucleic acid
ROS	reactive oxygen species
RT-PCR	reverse transcriptase polymerase chain reaction
SDH	succinate dehydrogenase
SDHA	succinate dehydrogenase, subunit A
SDHB	succinate dehydrogenase, subunit B
SDHC	succinate dehydrogenase, subunit C
SDHD	succinate dehydrogenase, subunit D
SMAD2, -4	Mad (*D. melanogaster)* homologs 2 and 4
SNP	single nucleotide polymorphism
T	thymine
TCAC	tricarboxylic acid cycle
TGFα	transforming growth factor α
TP53	gene encoding tumour protein 53 (p53)
TRC8	translocation in renal carcinoma on chromosome 8
TSC	tuberous sclerosis complex
ULM	uterine leiomyoma
ULMS	uterine leiomyosarcoma
UTR	untranslated region
UV	ultra violet radiation
VEGF	vascular endothelial growth factor
VHL	von Hippel-Lindau syndrome
WT	wild type
XP	xeroderma pigmentosum

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles, which are referred to the text by Roman numerals I-IV.

I Vanharanta S*, Buchta M*, McWhinney SR*, **Virta SK***, Peczkowska M, Morrison CD, Lehtonen R, Januszewicz A, Jarvinen H, Juhola M, Mecklin JP, Pukkala E, Herva R, Kiuru M, Nupponen NN, Aaltonen LA, Neumann HP, and Eng C (2004) Early-onset renal cell carcinoma as a novel extraparaganglial component of SDHB-associated heritable paraganglioma. *Am J Hum Genet* 74, 153-159.

II **Ylisaukko-oja SK**, Kiuru M, Lehtonen HJ, Lehtonen R, Pukkala E, Arola J, Launonen V, and Aaltonen LA (2006) Analysis of fumarate hydratase mutations in a population-based series of early onset uterine leiomyosarcoma patients. *Int J Cancer* 119, 283-287.

III **Ylisaukko-oja SK**, Cybulski C, Lehtonen R, Kiuru M, Matyjasik J, Szymanska A, Szymanska-Pasternak J, Dyrskjot L, Butzow R, Orntoft TF, Launonen V, Lubinski J, and Aaltonen LA (2006) Germline fumarate hydratase mutations in patients with ovarian mucinous cystadenoma. *Eur J Hum Genet* 14, 880-883.

IV Lehtonen HJ*, **Ylisaukko-oja SK***, Kiuru M*, Karhu A, Lehtonen R, Vanharanta S, Jalanko A, Aaltonen LA, and Launonen V. Stress induced expression of a novel variant of human *Fumarate hydratase (FH)*. Submitted.

* Equal contribution

Publication I is also included in the thesis of Sakari Vanharanta (Fumarate hydratase and succinate dehydrogenase in neoplasia, Helsinki 2006).

ABSTRACT

Hereditary leiomyomatosis and renal cell cancer (HLRCC) is a recently characterized cancer syndrome which predisposes to cutaneous and uterine leiomyomas as well as renal cell carcinoma (RCC). Uterine leiomyosarcoma (ULMS) has also been observed in certain Finnish HLRCC families. The predisposing gene for this syndrome, *fumarate hydratase (FH)*, was identified in 2002. The well-known function of FH is in the tricarboxylic acid cycle (TCAC) in the energy metabolism of cells. As *FH* is a novel cancer gene, the role of *FH* mutations in tumours is in general unknown. Similarly, the mechanisms through which defective *FH* is associated with tumourigenesis are unclear. The loss of a wild type allele has been observed in virtually all HLRCC patients tumours and the FH enzyme activities are either totally lost or remarkably reduced in the tissues of mutation carrier patients. Therefore, FH is assumed to function as a tumour suppressor.

Mutations in genes encoding subunits of other TCAC enzyme SDH have also been reported recently in tumours: mutations in *SDHB*, *SDHC*, and *SDHD* genes predispose to paraganglioma and pheochromocytoma.

In the present study, mutations in the *SDHB* gene were observed to predispose to RCC. This was the first time that mutations in *SDHB* have been detected in extra-paraganglial tumours. Two different *SDHB* mutations were observed in two unrelated families. In the first family, the index patient was diagnosed with RCC at the age of 24 years. Additionally, his mother with a paraganglioma (PGL) of the heart and his maternal uncle with lung cancer were both carriers of the mutation. The RCC of the index patient and the PGL of his mother showed LOH. In the other family, an *SDHB* mutation was detected in two siblings who were both diagnosed with RCC at the ages of 24 and 26 years. One of the siblings also suffered PGL. All these tumours showed LOH. Therefore, we concluded that mutations in *SDHB* predispose also for RCC in certain families.

Several tumour types were analysed for *FH* mutations to define the role of *FH* mutations in these tumour types. In addition, patients with a putative cancer phenotype were analysed to identify new HLRCC families. Three *FH* variants were detected, of which two were novel. One of the variants was observed in a patient diagnosed with ULMS at the age of 41 years. However, LOH was not detected in the tumour tissue. The FH enzyme activity of the mutated protein was clearly reduced, being 43% of the activity of the normal protein. Together with the results from an earlier study we calculated that the prevalence of *FH* mutations in Finnish non-syndromic ULMS is around 2.4%. Therefore, *FH* mutations seem to have a minor role in the pathogenesis on non-syndromic ULMS. Two other germline variants were detected in a novel tumour type, ovarian mucinous cystadenoma. However, tumour tissues of the patients were not available for LOH studies and therefore LOH status remained unclear. Therefore, it is possible that *FH* mutations predispose also for ovarian tumours but further studies are needed to verify this result.

A novel variant form of the *FH* gene (*FHv*) was identified and characterized in more detail. *FHv* contains an alternative first exon (1b), which appeared to function as 5' UTR sequence. The translation of *FHv* is initiated *in vitro* from exons two and three.

The localization of *FHv* is both cytosolic and nuclear, in contrast to the localization of *FH* in mitochondria. *FHv* is expressed at low levels in all human tissues. Interestingly, the expression was induced after heat shock treatment and in chronic hypoxia. Therefore, *FHv* might have a role e.g. in the adaptation to unfavourable growth conditions. However, this remains to be elucidated.

1 REVIEW OF THE LITERATURE

1.1 Cancer is a genetic disease

Cancer is a disease which is characterized by unregulated growth of cells. In fact, cancer is not one disease but a group of diseases, with different symptoms, origins, phenotypes, and prognoses. To date, approximately 110 different types of human cancer have been characterized (Weinberg 2007).

The aberrant growth that characterizes cancer is caused by mutations in the DNA of the cells. The accumulation of these mutations in the cell results in a state where the cell is unable to control its own growth and proliferation and becomes cancerous. Recently, it has been proposed that there are six features typical for almost all cancerous cells. These features include the self-sufficiency in growth stimulating signals, insensitivity to growth inhibiting signals, ability to avoid apoptosis and to replicate limitlessly, as well as the capacity to induce angiogenesis and to metastasize (Hanahan and Weinberg 2000). These features are consequences of the accumulation of mutations in the genes which regulate the proliferation, growth, differentiation, and programmed death of the cell. To date, over 290 cancer genes have been identified (for a rewiev, see Futreal et al. 2004). However, the number will probably increase in the near future. Recently, Sjöblom et al. (2006) investigated mutated genes in breast and colorectal cancers and detected 189 mutated genes in these tumours. The vast majority of the mutated genes had not previously been characterized as cancer genes. Cancer genes can be classified into two subgroups: oncogenes and tumour suppressor genes.

1.1.1 Oncogenes

Oncogenes were discovered for the first time in the 1970s when Stehelin and his colleagues perceived that a DNA sequence related to chicken sarcoma transforming virus is present also in the genome of normal chicken cells (Stehelin et al. 1976). However, the first human oncogene with a point mutation, *HRAS*, was not reported until 1982 (Reddy et al. 1982). Oncogenes are mutated forms of the cells' normal counterparts, proto-oncogenes. These genes typically regulate the growth of the cell in a promoting manner or the death of the cell in a diminishing manner. Oncogenes encode, for example, growth factors, growth factor receptors, transcription factors, or intracellular signalling proteins. Mutations in these genes lead to abnormally active function of the protein. The activation of proto-oncogenes can occur through point mutations, gene amplifications, or chromosomal translocations (for a review, see Bishop 1991). Oncogenes behave dominantly at the cellular level: an activating mutation in one of the two alleles is sufficient to promote tumourigenesis (for a review, see Bishop1991).

Over one hundred oncogenes have been identified thus far (Futreal et al. 2004). The *RAS* genes, *KRAS*, *HRAS*, and *NRAS*, are the most often mutated genes in human cancers (Giehl 2005, Bos 1989). *RAS* genes encodes proteins that act as intracellular signal transducers. Mutated forms of these proteins are constitutively activated. The majority of mutated oncogenes occur in somatic cancers, but a few exceptions exist. Germline mutations in the *MET* oncogene have been reported in patients diagnosed with hereditary papillary renal carcinoma (HPRC, OMIM 605074, Schmidt et al. 1997). *MET* encodes a receptor of the hepatocyte growth factor (HGF) (Bottaro et al. 1991).

Germline mutations in *RET* predispose to multiple endocrine neoplasia type 2 (MEN2, OMIM 171400, Mulligan et al. 1993). *RET* oncogene encodes a tyrosine kinase receptor. Germline mutations in the *KIT* oncogene predispose to familial gastrointestinal stromal tumour syndrome (GIST, OMIM 606764, Nishida et al. 1998) and KIT functions as a transmembrane tyrosine kinase. Oncogenic mutations in *MET*, *RET* or *KIT* leads to the constitutive activation of the corresponding receptor (Schmidt et al. 1997, Salvatore et al. 2000). Mutations in the *CDK4* oncogene predispose to familial malignant melanoma (FMM, OMIM 609048, Zuo et al. 1996). CDK4 is involved in the control of cell proliferation during the G1 phase in the cell cycle (Harbour et al. 1999).

1.1.2 Tumour suppressor genes

Tumour suppressor genes regulate the growth of the cells by inhibiting growth or promoting death (Kinzler and Vogelstein 1997). Products of these genes affect a wide variety of cellular functions and the general role of these proteins is to reduce the likelihood that the cell would escape normal growth and proliferation control and became cancerous. At the cellular level tumour suppressor genes are recessive, which means that both alleles must be mutated for the gene to be inactivated (Kinzler and Vogelstein 1997). The idea of biallelic inactivation of tumour suppressor genes was first proposed by Alfred Knudson in 1971, when he presented his famous two-hit hypothesis (Knudson 1971). He studied the kinetics of the appearance of unilateral or bilateral retinoblastoma and concluded that these conditions are related: mutations in both alleles of cancer predisposing gene are needed for tumourigenesis. In the inherited form of retinoblastoma one mutated allele is inherited and a single additional mutation is sufficient for tumour formation, while in the sporadic form of the disease two somatic events are needed. However, the fact that those two events occur in two copies of the *RB1* gene was understood only later (Cavenee et al. 1983, Friend et al. 1986).

Tumour suppressor genes can be divided into three subclasses according to their functions: gatekeepers, caretakers and landscapers (Kinzler and Vogelstein 1997, 1998). *Gatekeepers* are genes which are involved in the direct regulation of the proliferation, differentiation, or apoptosis of the cell. Biallelic inactivation of a gatekeeper gene could directly promote the growth of a tumour. This subgroup includes e.g. the two most common tumour suppressor genes, *RB1* and *TP53*, whose pathways are mutated in almost all tumours (for a review, see Knudson 2002). RB1 functions as a key regulator of the cell cycle. Inactivation of RB1 allows a cell enter to S-phase even if the DNA is unrepaired, which leads to abnormally high proliferation rates. Germline mutations in *RB1* predispose to sarcomas in addition to retinoblastoma of childhood (OMIM 180200). *TP53* encodes tumour protein 53 (p53) which, in turn, controls for example the quality of the DNA and arrests the cell cycle or induces apoptosis if the DNA is damaged. If p53 is inactivated, cells are able to proliferate even before the DNA has been repaired. Germline mutations in *TP53* cause Li-Fraumeni syndrome (OMIM 151623), in which patients have increased risk for sarcomas and breast cancer (Malkin et al. 1990). In addition, it has been estimated that *TP53* exists in the mutated form in half of all human tumours (Weinberg et al. 2007). *Caretakers*, in turn, maintain the integrity of the genome and regulate the number of mutations accumulating in the genome of a cell (Kinzler and Vogelstein 1997). This group includes genes such as *MHS2*, *MLH1* and *XP* which are involved, for example, in the recognition and repair of damaged DNA. The inactivation of a caretaker gene does not directly lead to growth promotion but to genomic instability, which results in increased mutation rates for all

genes, including tumour suppressors and oncogenes (Kinzler and Vogelstein 1997). *Landscapers* are genes which are involved in the maintenance of the cellular microenvironment (Kinzler and Vogelstein 1998). Mutations in these genes can enable the development of the tumour, for example, by offering suitable growth conditions via abnormal stromal cells.

The majority of the hereditary cancer syndromes are caused by inherited mutations in tumour suppressor genes (for a review, see Knudson 2002). While one allele of a tumour suppressor gene is inactivated in all cells of the individual, only one somatic alteration is needed to completely lose the function of the gene. Therefore, the risk of developing tumours is higher for patients with germline mutations and the onset of the disease usually occurs at younger age than in general population. Inherited mutations in tumour suppressor genes are usually small alterations, such as point mutations, or short deletions or insertions. In contrast, somatic inactivation of the second allele (loss of heterozygosity, LOH) occurs more commonly via larger alterations such as deletion of the whole chromosome or chromosome arm, or via mitotic recombination or gene conversion (Weinberg 2007).

1.1.3 Epigenetic and environmental factors

DNA mutations leading to alterations in the structure of oncogenes or tumour suppressor genes have been considered as the main factors in tumourigenesis. However, also epigenetic factors, which have no direct impact on the DNA sequence, could affect the activation or inactivation of cancer related genes. These epigenetic factors include non-coding or microRNAs, hypo- or hypermethylation of the DNA, post-translational modification of the histones, and remodeling of the nucleosomes (O'Donnell et al. 2005, for a review, see Ducasse and Brown 2006).

DNA methylation is considered as the most common epigenetic alteration associated with the carcinogenesis. Either hypomethylation or hypermethylation have been observed in all types of cancer cells examined thus far (for a review, see Ducasse and Brown 2006). DNA methylation occurs at cytosines of CpG-rich regions of the genome. The addition of methyl groups to the cytosines at a promoter region can prevent the binding of transcription factors or polymerase to the promoter, and further lead to the silencing of a tumour suppressor gene. In several studies, however, DNA hypermethylation appears to affect mainly genes that are already suppressed (Bird 2002). Conversely, absence of appropriate methylation at the promoter region of an oncogene can lead to the abnormal activation of the gene. The other epigenetic factors contribute at least to the transcription of genes, the maintenance and regulation of chromatin structure, and the stability of the genome (for a review, see Ducasse and Brown 2006).

In addition to genetic and epigenetic factors, several environmental factors are known to contribute to tumourigenesis. These factors include e.g. exposure to UV-radiation or carcinogenic chemicals, and tobacco smoking. However, environmental factors do not directly cause cancer but instead increase the mutation rate in the DNA. This results in accumulation of mutations and thus an increased risk of cancer.

1.1.4 Cancer is a multistep process

Tumourigenesis originates from the accumulation of mutations in the genome of a single cell through which the cell acquires a growth advantage over the surrounding cells. The process proceeds after additional mutations occur in the descendant cells leading these cells towards cancerous growth. This clonal and multistep process leads to the situation in which all cells of a tumour are descendants of a single ancestor cell. However, these cells within a tumour mass are not identical, since different mutations occur in different cells by chance during tumourigenesis (figure 1).

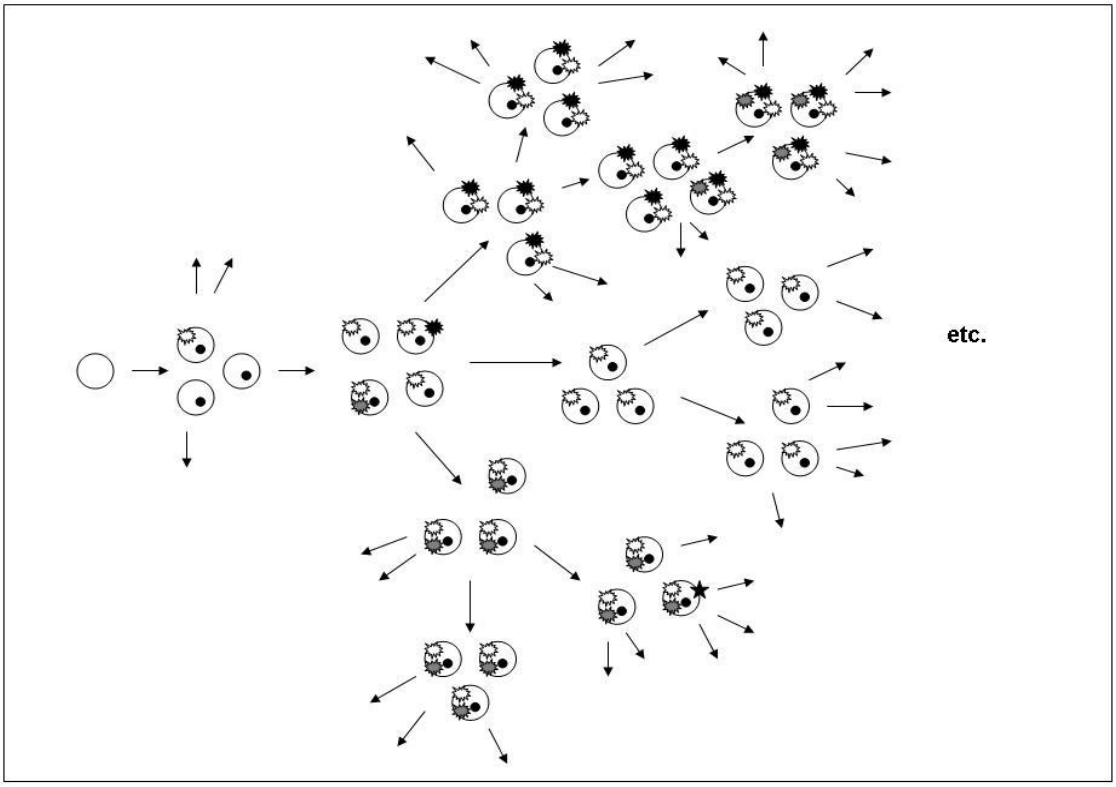

Figure 1. Clonal diversification through random mutations. All cells in a tumour are descendants of a single ancestor cell. However, these cells are not identical due to the random mutations occurring in each cell. Modified from Weinberg 2007.

The exact number of genetic alterations needed for carcinogenesis is unknown. It has been estimated that approximately four to seven functionally important changes would be needed for tumourigenesis (Renan 1993). Hanahan and Weinberg (2000) have suggested that changes in six distinct cellular regulatory pathways are needed to transform a normal human cell to a tumourigenic one (see section 1.1). They assume that virtually all tumours have to acquire alterations in these pathways, but the localization of the change along the pathway and the order of which these changes are acquired vary. However, Sjöblom et al. (2006) recently analysed over 13 000 protein encoding genes in breast and colorectal carcinomas and detected on average 90 mutated genes in individual tumours. On average, 11 genes per tumour were mutated at significant frequencies, and the remaining mutations were probably so-called passenger mutations, which do not contribute to tumourigenesis (Sjöblom et al. 2006). The accumulation of mutations in genes, which regulate the proliferation of the cell and

control the quality of the DNA leads to the instability of the genome of cancer cell. This results in aberrant karyotypes and abnormally high mutation rates observed in tumours and can explain the high number of passenger mutations.

The multistep progression of cancer has been studied in more detail in colorectal cancer, and a famous model called the adenoma-carcinoma sequence has been established (Fearon and Vogelstein, 1990, Kinzler and Vogelstein, 1996). According to this model, the progression of the disease begins from normal epithelium and proceeds to carcinoma through adenomatous stages, which are characterized by the accumulation of aberrations in the cells (Figure 2). These aberrations include inactivation of tumour suppressor genes and activation of oncogenes, as well as epigenetic factors such as DNA hypomethylation.

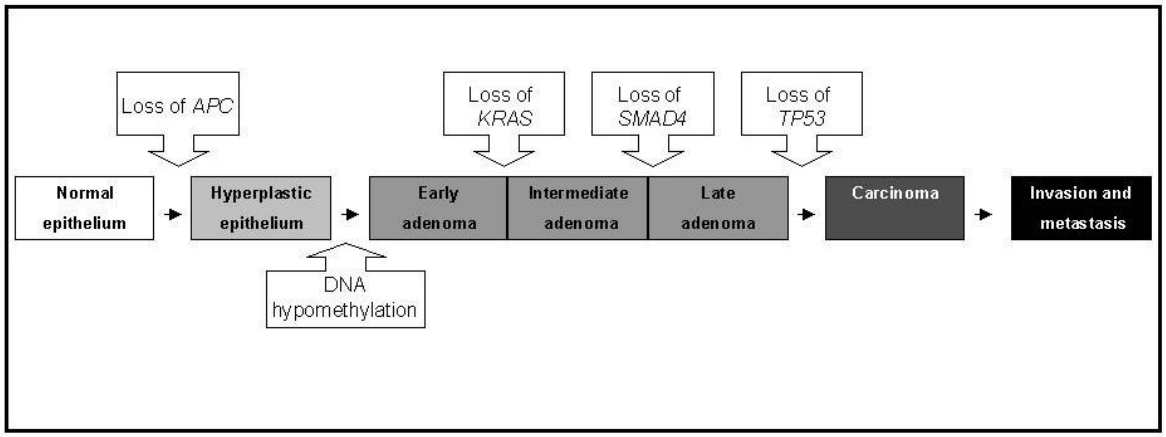

Figure 2. Adenoma-carcinoma sequence. The transformation of a normal epithelium to carcinoma occurs through adenomatous stages. This multistep process is characterized by inactivation of tumour suppressor genes (*APC*, *TP53*, *SMAD4*), activation of oncogenes (*KRAS*), and epigenetic factors such as DNA hypomethylation. Adapted from Kinzler and Vogelstein (1996) and Weinberg (2007).

1.1.5 Tumour stem cells

The very first idea of tumour stem cells was proposed in 1875, when Cohnheim suggested that cancer might arise from a small population of cells with stem cell like properties. Since then, several studies have indicated that a single tumour cell can be enough to generate a new tumour and accumulating evidence suggests the notion that tumour stem cells exist (for a review, see Polyak and Hahn 2006). According to this idea, tumour tissue contains a small population of stem cells with unlimited self-renewal capacity and tumourigenic potential. The great majority of tumour forming cells are fully or partially differentiated cells which can proliferate only a limited number of times and lack the ability to recreate a tumour. Results from several studies support the idea that tumours contain cells with stem cell properties: A small population of tumour cells which displays stem cell markers at their surface could transfer leukemia or breast cancer into mice, whereas thousands of fold higher numbers of cells lacking these cell surface markers could not (Dick et al. 1997, Al-Hajj et al. 2003). It has been proposed that all tumours origin from cancer stem cells and several models have been proposed for tumour stem cell development. In the first model, a mutation that affects asymmetric cell division occurs in a normal stem cell disrupting the regulation of asymmetric

division and leading to increased numbers of stem cells. These mutated stem cells acquire additional mutations and become cancer stem cells. In the second model, mutations have accumulated in one stem cell which is transformed to a malignant cancer stem cell. In the third model, a fully or partly differentiated cell acquires mutations which lead it toward a dedifferentiated state e.g. through an epithelial-mesenchymal transition. The cell mimics tumour stem cells but it is not a true cancer stem cell. While the origin of tumour stem cells is not unambiguous, evidence supporting all three hypotheses exists (for a review, see Polyak and Hahn 2006). In any case, if only a small number of tumour forming cells are capable of proliferating limitlessly, the mutation rate must be higher than earlier expected. However, the accumulation of mutations in cells leads to instability of the genome and further increases the mutation rate.

1.2 Renal cell carcinoma

Renal cell cancer (RCC) accounts for approximately 2-3% of all human cancer cases worldwide, and it appears that the incidence of RCC will increase (Kopper and Timar 2006, McLaughlin and Lipworth 2000). In Finland, approximately 750 new RCC cases are diagnosed annually, which accounts for 2.9% of all cancer cases (Finnish Cancer Registry 2005, Cancer statistics on the Finnish Cancer Registry web page last updated on 20th Sep 2006). As for cancer in general, RCC is not a single disease but a disease with several different morphological subtypes. The most common subtype is clear cell (conventional) RCC accounting for approximately 80% of cases. This is followed by papillary RCC (approximately 15% of cases), chromophobe RCC (approximately 5% of cases), collecting duct RCC (less than 1%), and unclassified RCCs (less than 2%; Kopper and Timar 2006). RCC occurs typically between 50 and 70 years of age, and is more common among men than women. The majority of RCC cases are sporadic, while hereditary forms of RCC account for less than 3% of all cases (for a review, see Kopper and Timar 2006).

1.2.1 Hereditary cancer syndromes with predisposition to renal cell carcinoma

1.2.1.1 Hereditary leiomyomatosis and renal cell cancer

In 1973, Reed and colleagues described for the first time a young patient with renal cell carcinoma, uterine leiomyosarcoma and cutaneous leiomyomas. The first degree relatives of this patient were also diagnosed with leiomyomas (Reed et al. 1973, Engelke and Christophers 1979). However, it took over two decades until these features were understood to be features of one syndrome. In 2001, a novel cancer predisposing syndrome was characterized with a predisposition to skin and uterine leiomyomas (ULM), RCC, and ULMS (Launonen et al. 2001). This syndrome was named hereditary leiomyomatosis and renal cell cancer (HLRCC, OMIM 605839), and the disease gene was genetically mapped to chromosome 1q42-q44 (Launonen et al. 2001). Concurrently, another research group described a similar syndrome with predisposition only to skin and uterine leiomyomas. This syndrome was named multiple cutaneous and uterine leiomyomata (MCUL, OMIM 150800), and the predisposing gene was mapped to the same chromosomal locus as HLRCC (Alam et al. 2001). In fact, these two syndromes were variants of the same disease with slightly different phenotypes. This was ascertained since the predisposing gene for both conditions was identified as *fumarate hydratase* (*FH*, *fumarase*) (Tomlinson et al., 2002). Since 2001, altogether

137 HLRCC families have been characterized worldwide. *FH* mutations have been reported in 89% of these families (122 out of 137 families) (Launonen et al. 2001, Kiuru et al. 2001, Kiuru et al. 2002, Alam et al. 2003, Martinez-Mir et al. 2003, Toro et al. 2003, Chan et al. 2005, Chuang et al. 2005, Kim 2005, Badeloe et al. 2006, Chuang et al. 2006, Lehtonen et al. 2006, Lehtonen et al. 2007, Varol et al. 2006, Wei et al. 2006).

HLRCC is inherited dominantly and LOH has been observed in almost all tumours, indicating that *FH* functions as a tumour suppressor gene (Kiuru and Launonen 2004, Tomlinson et al. 2002). The majority of patients carrying *FH* mutations are diagnosed with benign skin or uterine leiomyomas. The penetrance of skin or uterine leiomyomas among HLRCC patients with *FH* mutation is high, being approximately 85-89% (Alam et al. 2005c, Kiuru and Launonen 2004). Although these lesions are benign, they may cause difficult symptoms. There may be multiple skin leiomyomas and they may be painful. The uterine leiomyomas cause pain and bleeding, and are the most common reason for hysterectomy among women of fertile age.

In addition to benign leiomyomas, a minority of patients carrying *FH* mutations are predisposed to very aggressive RCC and ULMS. Both of these malign tumour types occur at especially young ages. The age of onset of the disease varies from 16 to 90 years in RCC and from 27 to 39 years in ULMS, while the median ages are 40.5 and 32, respectively (Lehtonen et al. 2006, Alam et al. 2005c). In addition to the exceptionally early onset, renal tumours in HLRCC families display unusual histologies. While the majority of the tumours display papillary type 2 histology, two tumours have been classified as collecting duct RCCs and one tumour as conventional (clear cell) RCC (Launonen et al. 2001, Alam et al. 2003, Toro et al. 2003, Chan et al. 2005, Badeloe et al. 2006, Lehtonen et al. 2007, Wei et al. 2006). In addition, the existence of renal tumours in HLRCC is typically unilateral, which is in contrast to the other hereditary RCC syndromes such as VHL and HPRC (see section 1.2.1.2). The penetrance of RCC and ULMS among *FH* mutation carriers is reduced, while RCC is observed in only 21% and ULMS in 2.5% of mutation positive HLRCC families (26 and 3 families out of 122 families, respectively) (Launonen et al. 2001, Kiuru et al. 2001, Kiuru et al. 2002, Alam et al. 2003, Martinez-Mir et al. 2003, Toro et al. 2003, Chan et al. 2005, Chuang et al. 2005, Kim 2005, Badeloe et al. 2006, Chuang et al. 2006, Lehtonen et al. 2006, Lehtonen et al. 2007, Varol et al. 2006, Wei et al. 2006). However, the risk of both RCC and ULMS is increased in the Finnish HLRCC families (Lehtonen et al. 2006). While RCC has been diagnosed in 71% of Finnish families (5 out of 7 families) compared to in 21% of HLRCC families worldwide, ULMS has been observed only in the Finnish families (3 out of 7 families, 43%). The sites or types of the *FH* mutations do not explain these differences in the occurrence of malignancies between populations, since mutation spectrum among families overlaps (see section 1.4.3). In addition to these characteristic tumours, several other tumour types have also infrequently been observed in HLRCC families. These tumour types include both malign and benign tumours such as breast carcinoma, prostate cancer, bladder carcinoma, hematological malignancies, testis leydig cell tumours, atypical uterine leiomyomas, ovarian and kidney cysts, and adrenal gland adenomas (Kiuru and Launonen 2004, Carvajal-Carmona et al. 2006, Lehtonen et al. 2006, 2007, Varol et al. 2006). However, at least some of these tumour types are propably observed by chance and are not caused by *FH* mutations.

1.2.1.2 Other hereditary syndromes with predisposition to renal cell carcinoma

Von Hippel-Lindau syndrome (VHL, OMIM 193300) is the most common hereditary RCC syndrome. The earliest reports concerning this syndrome were published in the beginning of the 20th century by Von Hippel (1904) and Lindau (1927). The current form of the VHL syndrome was characterized in 1964 by Melmon and Rosen. The most common features of VHL are retinal, cerebellar, and spinal hemangioblastoma occurring in approximately 60% of the patients (Maddock et al. 1996). Bilateral clear cell RCC occurs in approximately 25-45% of the patients and pheochromocytoma (PCC) in 15%. VHL has been classified as types 1 and 2 based on whether PCC exists (type 2) or not (type 1) (Neumann and Wiestler 1991). VHL type 2 has further been divided into type 2A (with PCC), and 2B (with PCC and RCC) (Brauch et al. 1995).

VHL syndrome is caused by heterozygous germline mutations in the *VHL* gene located at chromosome 3p25-p26 (Latif et al. 1993). Tumours of the patients typically display somatic loss of the wild type allele or hypermethylation at the *VHL* locus, indicating that *VHL* functions as a tumour suppressor gene (Crossey et al. 1994, Prowse et al. 1997). Different germline mutations in *VHL* cause different phenotypes: deletions, insertions and nonsense mutations are associated with VHL with RCC and without PCC, whereas missense mutations are associated with VHL with PCC (Chen et al. 1995, Zbar et al 1996). *VHL* mutations have also been observed in the majority (approximately 70%) of sporadic RCCs.

The VHL protein (pVHL) functions as a regulator of the critical transcription factor termed hypoxia-inducible factor1 (HIF1) (Iliopoulos et al. 1996, Maxwell et al. 1999). HIF1 is composed of two subunits, HIF1α and HIF1β. In normoxia, HIF1α is oxidized by prolyl hydroxylase. pVHL binds to the oxidized form of HIF1α and directs it into proteasomes for degradation. In hypoxia conditions, HIF1 is not oxidized and VHL is unable to bind it. Therefore, HIF1α levels increase in the cell and HIF1α is transported to the nucleus where it dimerizes with HIF1β and forms an active transcription factor HIF1. HIF1 evokes the expression of a cohort of target genes encoding, for example, growth factors involved in angiogenesis such as vascular endothelia growth factor (VEGF), platelet-derived growth factor (PDGF), and transforming growth factor α (TGFα). Mutations in *VHL* lead to inactive forms of pVHL, which are unable to bind HIF1α and direct it to degradation. This results in accumulation of HIF1α in the cell also in normoxia, and continuous expression of HIF1 target genes (Kim and Kaelin 2004).

Hereditary papillary RCC (HPRC, OMIM 605074) is characterized by the development of multiple, bilateral papillary type 1 renal cell tumours (Zbar et al., 1994, 1995). HPRC is inherited dominantly and is caused by mutations in the *MET* oncogene at chromosome 7q31-q34 (Schmidt et al. 1997). Duplications at the *MET* locus have also been observed in tumours (Zhuang et al. 1998). *MET* encodes a cell-surface tyrosine-kinase receptor for hepatocyte growth factor (HGF) (Bottaro et al. 1991). Duplications or mutations in *MET* lead to constitutive activation of the receptor, aberrant signalling, enhanced kinase activity, and increases in the invasive and metastatic properties of the cell (Giordano et al. 1997, Jeffers et al. 1997, 1998, Schmidt et al. 1997).

Birt-Hogg-Dubé syndrome (BHD, OMIM 135150) was characterized for the first time in 1977 by Birt and colleagues (Birt et al. 1977). BHD is inherited dominantly and is

characterized by renal tumours, hair follicle hamartomas, and spontaneous pneumothorax (Nickerson et al. 2002). The histology of renal tumours vary, including papillary RCC, clear cell carcinomas, and oncocytomas (Toro et al. 1999). BHD syndrome is caused by mutations in the gene encoding folliculin (*FLCN*) at chromosome 17p11 (Nickerson et al 2002). The function of *FLCN* is unknown.

Tuberous sclerosis complex (TSC, OMIM 191100) is a dominantly inherited syndrome reported for the first time in 1969 (Anderson and Tannen 1969). TSC is characterized by the presence of hamartomas in multiple organ systems. Central nervous system lesions cause epilepsy, learning difficulties, and behavioral problems. Many patients have renal lesions, usually angiomyolipomas, but renal cysts and RCC have also been reported (Crino et al. 2006). TSC is a genetically heterogeneous disease with two identified predisposing genes, *TSC1* at chromosome 9q34 and *TSC2* at chromosome 16p13 (Consortium TECTS 1993, van Slegtenhorst et al. 1997).

In addition to hereditary syndromes characterized by RCC, familial RCC could be associated with thyroid carcinoma syndrome *hyperparathyroidism-jaw tumour syndrome* (HPT-JT, OMIM 145001). HPT-JT syndrome is characterized by hyperparathyreoidism caused by parathyroid tumours, and the predisposing gene for this syndrome is *HRPT2* at chromosome 1q25-p32 (Carpten et al. 2002). RCC is also infrequently observed in patients diagnosed with *hereditary non-polyposis colorectal cancer* (HNPCC, OMIM 120435; Lynch and de la Chapelle 1999). The most common features of HNPCC are colorectal and endometrial cancer, but individuals with this syndrome also have increased risk of stomach, ovary, ureter, and small bowel cancers (Mecklin and Järvinen 1991, Lynch and de la Chapelle 1999). While HNPCC is caused mainly by mutations in DNA mismatch repair (MMR) genes *MHS2* and *MLH1* (mutations in these genes explain approximately 60% of HNPCC cases), mutations in other MMR genes, *PMS* and *MSH6*, have also been reported (Fishel et al. 1993, Leach et al 1993, Bronner et al. 1994, Nicolaides et al. 1994, Papadopoulos et al. 1994, Akiyama et al.1997, Miyaki et al.1997, Worthley et al. 2005). Hereditary RCC is also infrequently associated with *translocations in chromosome 3*. A family with clear cell RCC and a translocation t(3;8)(p14;q24) was reported in 1979 for the first time (Cohen et al. 1979). The disease was of early onset and dominantly inherited, and the occurrence of renal tumours was bilateral. The observed translocation produced a fusion of two genes, *FHIT* and *TRC8* (Ohta et al 1996, Gemmill et al 1998). However, the role of these genes in the tumourigenesis has remained controversial. Since 1979, several RCC cases with translocations at chromosome 3 have been reported (Kovacs et al. 1989, Schmidt et al.1995, Bodmer et al. 1998, 2002, Gemmill et al. 1998, Koolen et al. 1998, Kanayama et al. 2001, Melendez et al. 2003, Rodriguez-Perales et al. 2004, Foster et al. 2007).

1.3 Uterine leiomyosarcoma

Uterine leiomyosarcoma is a rare and highly malignant type of smooth muscle tumour accounting for only 1-3% of uterine malignancies. ULMSs are diagnosed mainly after menopause in women over 40 years of age (Barbieri and Andersen 1992). The genetic background and pathogenesis of ULMSs are poorly understood. ULMSs are typically characterized by numerous inconsequent chromosomal aberrations including losses of chromosomes 1p, 10q, 13q, 14q, and 22q, as well as gains of chromosomes 1, 8, 10, 17, and X (Packenham et al. 1997, Levy et al. 2000, Hu et al. 2001). However, a few

specific genetic changes have been observed in non-syndromic ULMSs, such as gene mutations in *TP53*, *PTEN* and *FH* (de Vos et al. 1994, Amant et al. 2002, Kiuru et al. 2002). It has also been presumed that ULMS could be a malign counterpart of benign uterine leiomyoma, and that 0.1% of uterine leiomyomas would progress to ULMS (Barbieri and Andersen 1992, Walker and Stewart 2005, Morton 1998). However, no chromosomal aberrations common to ULMS and uterine leiomyoma have been demonstrated in recent cytogenetic studies (Packenham 1997, Levy et al. 2000). Furthermore, in gene expression studies a number of genes have been observed to be differentially expressed in ULMS compared to uterine leiomyoma (Skubitz and Skubitz 2003). In addition to the exceptional occurrence of sporadic ULMSs, these tumours are infrequently observed as a part of the phenotype in some hereditary cancer syndromes.

As mentioned above, HLRCC in one of these hereditary syndromes characterized in part by early onset ULMS, but it has been observed only in Finnish HLRCC families (Kiuru and Launonen 2004). The only exception is a family with a patient diagnosed with ULMS, RCC and leiomyomas reported in 1973 by Reed and colleagues. In Finnish HLRCC families, ULMS has been diagnosed in 5 families out of 7 (71%) and the ages of the patients have been exceptionally young, varying from 27 to 39 years (Kiuru and Launonen 2004). Therefore, the risk of ULMS among *FH* mutation carriers is 71-fold when compared to the general population (Lehtonen et al. 2006). An *FH* mutation has also been detected in one patient with seemingly sporadic ULMS without a familial cancer history (Kiuru et al. 2002). However, the patient carried the mutant allele in the germline, and therefore the tumour was not purely somatic. In addition to ULMS, several HLRCC patients have been diagnosed with atypical uterine leiomyoma (Toro et al. 2003, Lehtonen et al. 2006). Atypical leiomyoma is a variant form of leiomyoma, which might sometimes be difficult to distinguish from leiomyosarcoma.

ULMS have also been observed in *Li-Fraumeni syndrome* (LFS, OMIM 151623, 609265), which is inherited dominantly and characterized by different early onset tumours, such as soft tissue sarcomas and osteosarcomas, breast cancer, brain tumours, leukemia, and adrenocortical carcinoma (Li and Fraumeni 1969, 1982). LFS is caused by mutations in the *TP53* gene at chromosome 17p13 (LFS1, OMIM 151623) or the *CHEK2* gene at chromosome 22q11 (LFS2, OMIM 609265) (Malkin et al. 1990, Bell et al. 1999).

1.4 Tricarboxylic acid cycle

The tricarboxylic acid cycle (TCAC, citric acid cycle, Krebs cycle) is a central pathway for oxidation of the acetyl groups of carbohydrates, lipids and proteins to carbon dioxide and water. The TCAC functions in aerobic conditions in the matrix of the mitochondria (figure 3). All human cells contain variable numbers of mitochondria, the double membrane-bounded cell organelle. The majority of the adenosine triphosphate (ATP), which is the 'molecular currency' of intracellular energy transfer, is produced in mitochondria via the TCAC and oxidative phosphorylation (Alberts et al. 1994). During each turn of the TCAC, three hydride ions are transferred to three NAD^+ molecules, and one pair of hydrogen atoms is transferred to an FAD molecule, when 3 NADH and 1 $FADH_2$ molecules are formed. These electron carriers transfer electrons to the electron transport chain (ETC), in which the electron carriers are oxidized yielding nine molecules of ATP. In the ETC, electrons are transferred from NADH and $FADH_2$ to CO_2 by a series of electron carriers, which is coupled with pumping of H^+ into

mitochondrial intermembrane space. The membrane potential is released through the fifth complex of respiratory chain, the ATP synthase, and utilized to phosphorylate ADP + P_i to ATP. ETC takes place in the inner membrane of mitochondria and consists of complexes I-IV. The respiratory chain consists of complexes I-V (figure 3). (Stryer 1999)

1.4.1 The role of succinate dehydrogenase and fumarate hydratase in TCAC

Succinate dehydrogenase (SDH, ETC complex II) is a nuclear-encoded enzyme of the TCAC which converts succinate to fumarate by oxidation (figure 3). In addition, SDH functions as a component of the ETC and has some specific redox properties to handle superoxides which are formed in oxidation-reduction processes (Carrel et al. 1990, Rustin et al. 2002). SDH is embedded in the inner membrane of mitochondria in contrast to the other enzymes of the TCAC, which are located in the matrix of the mitochondria. Thus, SDH forms a direct linkage between the TCAC and ETC of oxidative phosphorylation (Stryer 1999). SDH is a part of respiratory complex II (succinate-ubiquinone oxidoreductase) and consists of four different subunits named SDHA, SDHB, SDHC, and SDHD in order of decreasing molecular weight. Subunit A is a flavoprotein with a molecular weight of 70 kDa and contains the active site of the enzyme. Subunit B is a 27 kDa iron-sulfur protein in which the iron-sulfur cluster functions as an electron carrier (Carrel et al. 1990, Au et al. 1995). Subunits A and B together comprise the part of the enzyme that projects out from the inner membrane and is active in the citric acid cycle (Carrel et al. 2002). The whole enzyme complex is anchored into the inner membrane by integral membrane proteins, SDHC (15 kDa) and SDHD (12 kDa) (Parfait et al. 2000). SDHC and SDHD are also required for transferring electrons from SDH to ubiquinone, which is also called coenzyme Q (Rustin et al. 2002).

As SDH oxidizes succinate to fumarate in the TCAC, one hydrogen atom (H_2) is released. Covalently bounded FAD in the active site of SDHA functions as an acceptor of the released hydrogen. The reduced form, $FADH_2$, does not dissociate from the enzyme but two electrons are transferred directly from $FADH_2$ to the iron-sulfur cluster of SDHB. In the next stage, electrons are transferred from the iron-sulfur cluster to ubiquinone and further to the ETC (Stryer 1999). The reduced form of ubiquinone also functions as an antioxidant for biological membranes protecting them from oxidation (Ernster et al. 1995). Thus, ubiquinones reduced by SDH function as an antioxidant reservoir in the inner membrane of mitochondria (Rustin et al. 2002).

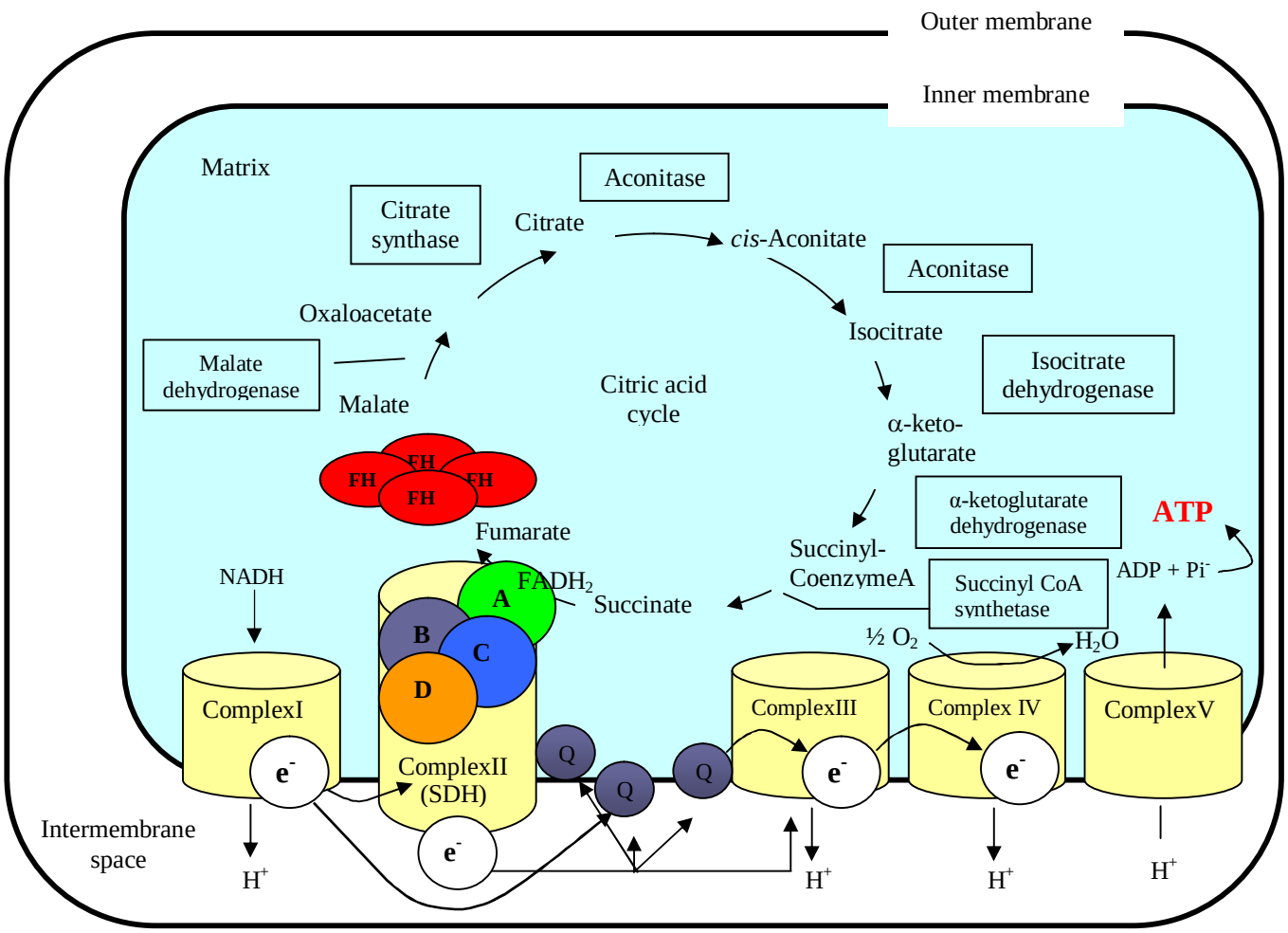

Figure 3. Tricarboxylic acid cycle and electron transport chain in mitochondria. The TCAC occurs in the matrix of the mitochondria. SDH (A, B, C, and D, complex II) participates in the TCAC and converts succinate to fumarate. FH, in turn, hydrates fumarate into malate. The other enzymes of TCAC are placed in boxes. SDH participates also in the ETC in the inner membrane, and forms a direct link between the TCAC and ETC. Electrons (e^-), which are formed in the TCAC, are bound to $FADH_2$, and transferred from SDHA via SDHB, SDHC, and SDHD through ubiquinone (Q) to complex III. Hydrogen ions (H^+) are pumped into intermembrane space. The membrane potential is released through the complex V and ATP is formed. Modified from Rustin et al. (2002).

Fumarase hydratase (FH, fumarase) is a nuclear encoded enzyme which functions in the TCAC immediately after SDH, and hydrates fumarate into malate (figure 3). The functional enzyme is a homotetramer consisting of four similar subunits with a molecular weight of 50 kDa each. In addition to this mitochondrial form, a cytosolic form of FH also exists. The known functions of the cytosolic form are fumarate metabolisms in the amino acid metabolism and urea cycle. Both the mitochondrial and cytosolic forms of the FH protein are identical (Sass et al. 2003). The exact mechanism through which this dual targeting is accomplished in human cells is not clear. However, results from localization studies in yeast have suggested that all translation products of

FH are first targeted and processed in the mitochondria. Thus, after the removal of the mitochondrial signal peptide, cytosolic FH returns to the cytoplasm (Knox et al. 1998, Sass et al. 2001, 2003).

1.4.2 Paragangliomas and pheochromocytomas result from defective SDH

Defects of SDH are relatively rare in humans, which indicates its indispensability for the function of mitochondria (Rustin et al. 2002). Inherited mutations in different subunits of SDH cause different diseases. Heterozygous mutations of three subunits, *SDHB*, *SDHC*, and *SDHD*, cause tumour predisposing syndromes, while homozygous mutations in *SDHA* cause severe neurological symptoms (Rivner et al. 1989, Bourgeron et al. 1995, Baysal et al. 2000, for a review, see Eng et al. 2003). Mutations in any SDH subunits result in decreased enzyme activity. In SDH deficiencies caused by mutations in *SDHA*, residual activities are quite high in tissues, manifesting 25-50% of control mean enzyme activities. This suggests that homozygous null mutants are lethal. In contrast to tissues with homozygous *SDHA* mutations, tumour tissues with mutations in both alleles of *SDHB*, *SDHC*, or *SDHD* have measured activities of almost zero (Bourgeron et al. 1995, Birch-Machin et al. 2000, Parfait et al. 2000). *SDHB*, *SDHC*, and *SDHD* have been thought to act as tumour suppressor genes and LOH has been frequently observed in tumours (for a review, see Baysal 2002).

The roles of mutated *SDHB, SDHC*, and *SDHD* in neoplasias were identified quite recently (Baysal et al. 2000, Astuti et al. 2001, Carrel et al. 2002, Niemann and Muller 2000). Mutations in *SDHB* have been reported to cause classical autosomal inherited neoplasia syndromes with pheochromocytomas as well as head and neck paragangliomas (Astuti et al. 2001). Additionally, numerous sporadic PCCs with *SDHB* mutations have been reported (Cascon et al. 2002a, van Nederveen et al. 2006). Mutations in *SDHD* cause hereditary and sporadic PGL (OMIM 168000) as well as sporadic PCC (Carrel et al. 2002, Cascon et al. 2002b, Neumann et al. 2002). *SDHD* mutations have been reported also in one case of midgut carcinoids (Kytola et al. 2002). Mutations in *SDHC* are clearly more infrequent than mutations in *SDHB* or *SDHD*, since only four cases of PGL with *SDHC* mutations have been reported to date (Niemann & Muller 2000, Niemann et al. 2003, Bayley et al. 2006, Schiavi et al. 2006).

PCCs are rare and usually benign adrenal medullary tumours, whereas PGLs are extra-adrenal pheochromocytomas, located in the abdominal and mediastinal sympathetic ganglions (OMIM 171300). Approximately 10% of PCCs and up to 50% of PGLs are familial (Astuti et al. 2001). It has been estimated that approximately 50% of familial PCCs and 20% of familial and 3% of sporadic PGLs are caused by *SDHB* mutations (Astuti et al. 2001, Baysal et al. 2002, Cascon et al. 2002a). Baysal et al. (2002) has also estimated that *SDHD* mutations might be responsible for approximately 50% of familial PGLas and 5% of sporadic cases. Interestingly, PGLs caused by mutations in *SDHD* are inherited paternally, whereas there is no evidence of parent-of-origin effects for *SDHB* and *SDHC* mutations (Baysal et al. 2000, Niemann et al. 2003).

Mutations in SDHA cause encephalomyopathies with distinct phenotypes (Bourgeron et al. 1995, Birch-Machin et al. 2000, Parfait et al. 2000, Van Coster et al. 2003, Horvath et al. 2006). These syndromes include infantile encephalopathies, such as Leigh syndrome (OMIM 256000), as well as hypertrophic cardiomyopathy, with or without skeletal muscle myopathy, and late-onset neurodegenerative syndrome with optic

atrophy, ataxia and myopathy (Leigh 1951, Burgeois et al. 1992, Bourgeron et al. 1995, Birch-Machin et al. 1996, Reichmann and Angelini 1994).

1.4.3 HLRCC and FH deficiency are consequences of defective *FH*

Similarly to SDH, mutations in *FH* cause two different phenotypes: a cancer syndrome called HLRCC (see section 1.2.1.1) and a childhood encephalopathy called FH deficiency (FHD, OMIM 606812). HLRCC is characterized by benign and malign tumours, whereas FHD is characterized by developmental delay, hypotonia, encephalopathy and fumaric aciduria, and patients typically survive only a few months (Gellera et al. 1990). HLRCC is caused by heterozygous germline *FH* mutations, whereas FHD results from homozygous (or compound heterozygous) mutations (Tomlinson et al. 2002, Bourgeron et al. 1994, Coughlin et al. 1993). Pathogenic mutations in *FH* result in decreased enzyme activity: the activities in homozygous FHD patient tissues and in HLRCC patient tumours are close to zero, whereas in the normal tissues of HLRCC patients and in heterozygous parents of FHD patients the activities are approximately 50% of the normal values (Gellera et al. 1990, Coughlin et al. 1998, Alam et al. 2003, Tomlinson et al. 2002, Pithukpakorn et al. 2006).

Both of these syndromes occur rarely. So far, a total of 137 distinct HLRCC families have been reported worldwide (Launonen et al. 2001, Kiuru et al. 2001, Kiuru et al. 2002, Alam et al. 2003, Martinez-Mir et al. 2003, Toro et al. 2003, Chan et al. 2005, Chuang et al. 2005, Kim 2005, Badeloe et al. 2006, Chuang et al. 2006, Lehtonen et al. 2006, Lehtonen et al. 2007, Varol et al. 2006, Wei et al. 2006). FHD, in turn, has been diagnosed in 20 unrelated families (Bourgeron et al. 1994, Gellera et al. 1994, Rustin et al. 1997, Coughlin et al. 1998, Alam et al. 2003, Remes et al. 2004, Loeffen et al. 2005, Pollard et al. 2005b, Deschauer et al. 2006, Maradin et al. 2006, Phillips et al. 2006, Zeng et al. 2006). Altogether, 88 different *FH* mutations have been reported. Seventy-one of these mutations have been reported in tumours characteristic of HLRCC, 18 in FHD patients, and 6 in both of these syndromes (table 1). In addition to tumours detected in HLRCC families, *FH* mutations have been reported infrequently in other tumours (Kiuru et al. 2002, Lehtonen et al. 2004, Carvajal-Carmona et al. 2006). The mutations observed in both syndromes are spread uniformly along the gene and the mutation spectrums overlap (table 1). Point mutations in four different codons, R58, K187, R190, and H275, as well as a three base pi insertion 435insAAA have been observed in both HLRCC and FHD families (Gellera et al. 1994, Chuang et al. 2005, Coughlin et al. 1998, Tomlinson et al. 2002, Toro et al. 2003, Pollard et al. 2005b, Deschauer et al. 2006, Wei et al. 2006). However, the phenotypes of these syndromes are totally distinct. While the penetrance of leiomyomas among HLRCC patients is high, being approximately 85-89%, the heterozygous parents or siblings of FHD patients do not typically display the features of HLRCC (Alam et al. 2003, Remes et al. 2004, Deschauer et al. 2006). Leiomyomas have been observed in only three heterozygous relatives of FHD patients in two unrelated FHD families out of 20 families examined to date (Tomlinson et al. 2002, Maradin et al. 2006).

Table 1. *FH* mutations reported to date. Altogether, 88 distinct mutations have been reported, of which 71 have been observed in tumour types characteristic of HLRCC, 18 in FHD patients, and 6 in both syndromes. In addition, mutations in several other tumour types have been reported (marked as superscript). No correlations between phenotypes and type or site of *FH* mutation were observed.

Exon	Mutation	Skin/uterine Leiomyoma	RCC	ULMS	FHD
All	Whole gene deletion	Tomlinson et al. 2002 Alam et al. 2005a			
1	Q4X	Tomlinson et al. 2002 Chuang et al. 2005			
1	R8E[1]*				
1	1bp del, L17fsX17	Tomlinson et al. 2002			
1	1bp ins, K37fsX	Wei et al. 2006	Wei et al. 2006		
1	2bp del, V41fs*		Tomlinson et al. 2002		
Intron 1	IVs1+1G>C	Wei et al. 2006			
2	R58X[6]	Tomlinson et al. 2002 Varol et al. 2006	Wei et al. 2006		Pollard et al. 2005b
2	R58P	Chan et al. 2005	Chan et al. 2005		
2	N64T[3]	Tomlinson et al. 2002 Wei et al. 2006			
2	A74P	Tomlinson et al. 2002			
2	1bp del, N78fsX85	Badeloe et al. 2006	Badeloe et al. 2006		
Intron 2	IVS2+1 delG	Badeloe et al. 2006			
3	L89S	Wei et al. 2006	Wei et al. 2006		
3	H92R	Chuang et al. 2005			
3	1bp del, V97fsX	Toro et al. 2003			
3	S102X		Wei et al. 2006		
3	S115I	Martinez-Mir et al. 2003 Chuang et al. 2005			
3	R117G	Wei et al. 2006			
3	P131R				Alam et al. 2003 Zeng et al. 2006
3	H137R	Tomlinson et al. 2002			
3	Q142R	Tomlinson et al. 2002 Chuang et al. 2005			
3	Q142X	Martinez-Mir et al. 2003			
3	Q142K	Badeloe et al. 2006			
Intron 3	IVS3+1 G>A	Badeloe et al. 2006			
4	S144L	Toro et al. 2003			
4	N145S	Toro et al. 2003			
4	P149L	Chuang et al. 2005			
4	M152T	Toro et al. 2003			
4	H153R		Kiuru et al. 2001	Kiuru et al. 2002	
4	2bp del, E181fsX205[2]	Kiuru et al. 2002	Tomlinson et al. 2002	Tomlinson et al. 2002	
4	I186T	Alam et al. 2003			
4	3bp del I186	Tomlinson et al. 2002			
4	K187R	Tomlinson et al. 2002 Toro et al. 2003 Chuang et al. 2005			Coughlin et al. 1998
4	R190L	Toro et al. 2003 Chuang et al. 2006	Chuang et al. 2006		
4	R190H	Tomlinson et al. 2002 Wei et al. 2006 Chuang et al. 2005	Toro et al. 2003 Wei et al. 2006		Gellera et al. 1994
4	R190C	Chuang et al. 2005	Wei et al. 2006		Rustin et al. 1997
4	A196T*	Lehtonen et al. 2004			
intron 4	IVS4+3 A>G*	Lehtonen et al. 2004			
5	R233H				Loeffen et al. 2005
5	G239V	Tomlinson et al. 2002			
5	L240X*			Kiuru et al. 2002	
6	2bp del, L260fsX	Toro et al. 2003			
6	P261fsX	Toro et al. 2003			
6	A265T				Coughlin et al. 1998
6	N267Y	Alam et al. 2005a			
6	F269C				Coughlin et al. 1998
6	H275Y	Chuang et al. 2005 Toro et al. 2003	Toro et al. 2003		Deschauer et al. 2006
6	4bp del, 1081del4	Chuang et al. 2005			
6	V279D	Toro et al. 2003			
6	M285R	Tomlinson et al. 2002			
6	T287P	Kim 2005			

Continued from the previous page

6	T287P	Chuang et al. 2006			
6	L292P	Toro et al. 2003			
6	N297D	Toro et al. 2003			
6	N297K	Wei et al. 2006			
6	R300X	Tomlinson et al. 2002	Tomlinson et al. 2002	Tomlinson et al. 2002	
6	E312K	Alam et al. 2003			
6	4bp del, N318fsX	Martinez-Mir et al. 2003			
6	N318K	Alam et al. 2003	Alam et al. 2003[4]		
6	E319Q				Bourgeron et al. 1994
6	S322G	Toro et al. 2003 Wei et al. 2005			
6	S323N	Alam et al. 2003			
intron 6	905G>A, splice	Chuang et al. 2005			
7	P326S				Maradin et al. 2006
7	N330S		Lehtonen et al. 2007[5]		
7	Q333P				Remes et al. 2004 Phillips et al. 2006
7	2bp ins, E335fsX	Toro et al. 2003	Toro et al. 2003		
7	A342D	Wei et al. 2006			
7	3bp del, V344del Q343H				Maradin et al. 2006
7	6bp del, delA350-V351	Chuang et al. 2005			
7	V351L	Martinez-Mir et al. 2003 Chuang et al. 2005			
7	G354R	Alam et al. 2003			
7	H360C				Phillips et al. 2006
8	S376P	Wei et al. 2006	Wei et al. 2006		
8	D383V				Coughlin et al. 1998
8	1bp del, T388fsX	Toro et al. 2003	Toro et al. 2003		
8	Q396P	Wei et al. 2006	Wei et al. 2006		
8	E404X	Badeloe et al. 2006			
8	1bp del, L406fsX410	Tomlinson et al. 2002			
9	M411I[3]				
9	1bp del, I413fsX449	Badeloe et al. 2006			
9	Y422C	Toro et al. 2003			
9	3bp ins, 435insK				Gellera et al. 1994 Coughlin et al. 1998 Deschauer et al. 2006 Rustin et al. 1997
9	S437fsX	Toro et al. 2003			
9	1bp del, G447fsX	Toro et al. 2003	Toro et al. 2003[7]		
9	W458X				Coughlin et al. 1998
9	L464P	Alam et al. 2003			
9	3bp ins, 477insK				Loeffen et al. 2005

[1] Soft tissue sarcoma, Kiuru et al. 2002
[2] Breast carcinoma, bladder carcinoma, Tomlinson et al. 2002
[3] Leydig cell tumour of testis, Carvajal-Carmona et al. 2006
[4] Renal collecting duct cancer (CDC)
[5] Bilateral, papillary type 2 and conventional RCC
[6] Ovarian cysts, Varol et al. 2006
[7] Papillary type 2 and collecting duct RCC
* Somatic mutation

1.4.4 Tricarboxylic acid cycle and cancer

It is not completely clear how defects in the TCAC lead to cancer formation. However, defects in SDH or FH result in decreased enzyme activities and prevent the normal function of the TCAC leading to the accumulation of succinate and fumarate in cells (Gellera et al. 1990, Coughlin et al. 1998, Alam et al. 2003, Tomlinson et al. 2002, Pithukpakorn et al. 2006, Bourgeron et al. 1995, Birch-Machin et al. 2000, Parfait et al. 2000, Selak et al. 2005, Pollard et al. 2005b). Several hypotheses of how this phenomenon leads to tumourigenesis have been proposed (Eng et al. 2003, Pollard et al. 2003, Gottlieb and Tomlinson 2005). These include, for example, accumulation of rective oxygen species (ROS) and oxidative stress, aberrant energy metabolism,

activation of glycolysis, mitochondrial dysfunction and defective apoptosis, activation of the hypoxia pathway in normoxia (pseudo-hypoxia), and the unknown function of the cytosolic form of FH (Eng et al. 2003, Pollard et al. 2003, Kiuru and Launonen 2004, Gottlieb and Tomlinson 2005, King et al. 2006). Currently, the strongest evidence has been for pseudo-hypoxia, the activation of the HIF-pathway in normoxia (King et al. 2006, Briere et al. 2006). Actually, the overexpression of HIF1 and its target genes have been observed in both SDH and FH deficient cell lines (Isaacs et al. 2005, Pollard et al. 2005b, Selak et al. 2005). Recently, accumulation of fumarate or succinate in cells due to defective FH or SDH have been shown to inhibit HIF1 prolyl hydroxylation in several independent studies (Selak et al. 2005, Pollard et al. 2005b, Koivunen et al. 2006). Therefore, fumarate and succinate seem to function as intracellular messengers between mitochondria and cytoplasm. The inhibition of prolyl hydroxylases (PHDs) leads to the stabilization of HIF1 and further to the increased expression of HIF1-regulated genes such as VEGF. Tumours with *SDHB*, *SDHD* and *FH* mutations have been reported to be highly vascularized, indicating activation of the HIF1 pathway (Gimenez-Rogueplo 2001, Pollard et al. 2005a).

2 AIMS OF THE STUDY

This study aimed to define the role of two mitochondrial enzymes, SDH and FH, in human tumourigenesis. More detailed aims of each study were as follows:

I) To define the role of mutations in *SDH* genes in early onset renal cell carcinoma.

II) To define the role of *FH* in early onset uterine leiomyosarcoma.

III) To identify new HLRCC families and further define the spectrum of tumours related to defective *FH* function.

IV) To characterize the structure and function of a novel variant form of the *FH* gene.

3. MATERIALS AND METHODS

3.1 Patient materials

3.1.1 Renal cell carcinomas (I)

Early onset RCCs (diagnosed between 15 and 34 years of age) of any histological type were sought from the Finnish Cancer Registry. The Finnish Cancer Registry is the nationwide and population-based database, which contains information of all cancer cases diagnosed in Finland since 1953. Therefore, the registry provides a unique opportunity to collect cancer related data and population based materials. Patients younger than 15 years of age were omitted due to the abundant occurrence of Wilms tumour during childhood. In addition, cases diagnosed before the year 1985 were excluded due to poor quality of DNA and problematic handling of old paraffin-embedded tissues. The search revealed 244 unrelated patients. The pedigrees were then expanded through the Finnish Population Registry, and the cancer history of the families was documented through the Finnish Cancer Registry and patient records. After these searches, one family with a potential family history (Fam1) was selected for further analyses. This family included a proband diagnosed with clear cell RCC at the age of 28 years and his mother diagnosed with malignant paraganglioma (PGL) of the heart at the age of 55 years. In addition, two maternal uncles of the proband were diagnosed with small-cell lung cancer and pancreatic carcinoma at the ages 55 and 71 years, respectively. Another family (Fam2) was ascertained through the search for RCC patients from the Population-based registry of the Freiburg-Warsaw-Columbus Pheochromocytoma Study Group. Fam2 contained two siblings diagnosed with RCCs of solid histology at the ages of 24 and 26 years. Both patients had also suffered PGL. In addition, 95 sporadic RCC tumours of various histologies were selected for mutation analyses (table 2). The collection and the documentation of the samples were performed in accordance with the institutional review boards for human subjects' protection of the University of Helsinki, the Ohio State University, the University of Freiburg, and the Institute of Cardiology of Warsaw.

DNA extraction from the paraffin-embedded tumour and normal tissues was carried out using deparaffinization with xylene, washing with ethanol, treatment with proteinase K, and extraction with phenol and chloroform (Kannio et al. 1996; studies I-IV).

Table 2. Histologies of sporadic RCC tumours analysed in study I.

Tumour histology	**N**	**Population**
Clear cell		
of which early onset (under 50 years)	35	American
diagnosed at any age	30	Finnish
Papillary	3	Finnish
Granular cell	2	Finnish
Mixed papillary / clear cell	1	Finnish
Mixed clear cell / solid histology	1	Finnish
Oncocytic papillary	9	Finnish
Oncocytoma	14	Finnish
Total	**95**	

3.1.2 Uterine leiomyosarcomas (II)

The purpose of study II was to investigate early onset ULMS, and therefore we searched the Finnish Cancer registry to build a population based material of ULMS. The search revealed altogether 392 cases diagnosed between the years 1984 and 2003. Most of the patients were diagnosed at ages above 50 years. A total of 81 cases were diagnosed at less than 46 years of age fulfilling the adjusted criteria for early onset. The majority of these cases were diagnosed at ages between 41 and 45 years (45/81, 56%) and 36 and 40 years (26/81, 32%). Only ten patients (10/81, 12%) were diagnosed under 35 years of age. Tumours originated most often from the uterine corpus (57 cases out of 81, 70%) and showed grade 1 (n=28) or 2 (n=29). The details of the material are presented in table 3.

A pathologist evaluated the proportion of tumour tissue in paraffin-embedded samples and only samples displaying at least 50% tumour tissue were accepted for DNA extraction and further analyses. The appropriate tumour tissue samples were available from 67 individuals. In addition, 280 samples obtained from the Finnish Red Cross were used as healthy population controls. The study was approved by the ethics committee of the Helsinki University Central Hospital and the National Authority for Medicolegal Affairs.

Table 3. Clinical data of the early onset ULMS samples

Age at diagnosis	N	Died of the disease	Site of tumour	N	Grade of tumour	N
41 - 45	45	12	Corpus	57	1	28
36 - 40	26	3	Fundus	2	2	29
31 - 35	5	0	NUD	22	3	8
<30	5	3			Unknown	16
Total	**81**	**18**		**81**		**81**

NUD, *non ultra descriptus*, undefined

3.1.3 Patient material to study putative *FH* mutation related phenotypes (III)

A total of 150 samples were included in this study. DNA of 89 samples was extracted from blood lymphocytes of patients diagnosed with RCC, skin leiomyomas or ovarian tumours. These samples were collected from Poland. In addition, 13 Finnish ovarian mucinous cystadenocarcinoma samples and 48 Danish bladder carcinoma samples were included. The DNA of ovarian tumours and bladder carcinomas were extracted from fresh frozen tumour tissues. Details of all samples are shown in table 4. The patient materials were collected either anonymously or following informed consent and the study was approved by the Ethics Committee of the Helsinki University Central Hospital and the National Authority for Medicolegal Affairs.

Table 4. Detailed description of the patient material analysed in study III

Samples	N	Tissue	Population
Tumors in probands			
Papillary renal cell cancer	24	Blood lymphocyte	Polish
Single skin leiomyomas	5	Blood lymphocyte	Polish
Ovarian tumours			
Musinous cystadenoma	33	Blood lymphocyte	Polish
Musinous cystadenocarcinoma	22	Blood lymphocyte	Polish
Serous cystadenoma	5	Blood lymphocyte	Polish
	89		
Tumour samples			
Bladder carcinoma	48	Fresh frosen tumour tissue	Danish
Ovarian carcinoma			
Mucinous cystadenocarcinoma	13	Fresh frosen tumour tissue	Finnish
	61		
Total	**150**		

3.1.4 Patient and cell line material for mutation analyses of *FHv* (IV)

In study IV, altogether 139 human DNA samples were analysed for *FH* exon 1b mutations. The material included human tumour cell lines, non-syndromic HLRCC-associated tumour samples, HLRCC patients without known second hit mutations and blood samples from *FH* mutation negative patients. The material is described in detail

in table 5. The collection and use of the patient material was approved by the National authority for Medicolegal Affairs and the Ethics Committee of the Department of Medical Genetics, University of Helsinki.

Table 5. Details of the material used in study IV

Samples	N	Tissue
Non-syndromic HLRCC associated tumours		
Renal cell carcinoma	43	Tumour
Uterine leiomyomas	39	Tumour
Uterine leiomyosarcoma	17	Tumour
	99	
HLRCC tumours without second hit	**9**	Tumour
FH mutation negative patients		
Early onset RCC	6	Blood
RCC or leiomyomatosis	4	Blood
	10	
Human cell lines		
Renal cell carcinoma	12	Cell line
Prostate cancer	4	Cell line
Sarcoma	5	Cell line
	21	
Total	**139**	

3.1.5 Other materials used in study IV

To determine *FHv* mRNA expression in different tissues, two human multiple tissue cDNA panels (Human MTC^{TM} Panel 1 and Human Fetal MTC^{TM} panel, BD Biosciences Clontech, Palo Alto, CA) were used. All cDNA constructs, which were used to determine the initiation of transcription, were cloned in both pCi-neo Mammalian Expression Vector (Promega Corporation, Madison, US) and pEGFP-N3 vector (BD Biosciences Clontech, Mountain View, CA). HEK293 (CRL1573, ATCC), HTB115 (SK-UT-1B, ATCC) and HeLa (CCL-2, ATCC) cell lines were used in cell line experiments.

3.2 Analysis methods

3.2.1 Mutation analyses of SDH (I)

The protein coding regions and exon-intron boundaries of *SDHA*, *SDHB*, *SDHC*, and *SDHD* were analysed by direct sequencing. The oligonucleotide primers for *SDHB*, *SDHC* and *SDHD* were obtained from the literature (Astuti et al., 2001, Baysal et al., 2000) whereas primers for *SDHA* were designed by the Primer3 program (Rozen and Skaletsky 2000).

PCR amplification of the fragments were performed in 50 µl reactions containing 100 ng of genomic DNA, 1 x GeneAmp PCR Buffer (Applied Biosystems, Foster City, CA), 300 µM of each dNTP (Finnzymes, Espoo, Finland), 1 µM of both primers (Sigma-Genosys, Cambridge, UK), 2.5 units of Ampli*Taq* Gold DNA polymerase (AB), and 1.5mM $MgCl_2$. PCR cycling conditions were as follows: denaturation at 95°C for 10 min was followed by 34 cycles of denaturation at 95°C for 45 s, annealing at 56 to 62°C for 1 min and elongation at 72°C for 1 minute, and final extension at 72°C for 10 minutes.

Next, the PCR products were verified by agarose gel electrophoresis and purified using the QIAquick spin PCR purification kit (QIAGEN, Valencia, CA). The sequencing reactions were performed using BigDye 3 termination chemistry (AB) according to the manufacturer's instructions. Electrophoresis was performed on an ABI3100 Genetic Analyzer (AB). The sequence chromatograms were analysed using the Multiple Alignment General Interface lineage program (MAGI), which was available on the web page of the UK Human Genome Mapping Project Resource Centre.

3.2.2 Mutation analyses of *FH* (II, III, IV)

Mutation analyses of exons and flanking intronic sequences were performed mainly by direct genomic sequencing. The PCR conditions and oligonucleotide primers for *FH* exons 1-10 were published earlier by Kiuru and colleagues (2002). Oligonucleotide primers for exon 1b were designed using the Primer3 program (Rozen and Skaletsky 2000). The primer sequences were 5'-GGC TGT CAG AGA GGG TCC TA-3' for the forward and 5'-TAC GGG GGA AAC CAT AGT CA-3' for the reverse primer. After amplification, PCR products were verified by agarose gel electrophoresis and purified using the ExoSAP-IT PCR purification kit (USB Corporation, Cleveland, Ohio, USA). The sequencing reactions were performed using the Big Dye Terminator v.3.1 kit (Applera Corporation, Norwalk, CT) according to the manufacturer's instructions. Electrophoresis was performed using an ABI3730 DNA sequencer (Applera Corporation) according to the manufacturer's instructions. The sequence chromatograms were analysed using either Chromas 2.21 (Technelysium Pty Ltd, Queensland, Australia) or EditView 1.0.1 software (AB).

FH mutation analyses of 48 bladder carcinoma samples (III) and 280 healthy controls (II) were performed by DHPLC. Amplification of the 10 *FH* exons was performed as described earlier by Lehtonen and colleagues (2003). After amplification, PCR reactions of two samples were pooled together and denatured at 95°C for three minutes, after which fragments were reannealed by reducing the temperature 0.5°C per 30 seconds for 45 minutes. The DHPLC heteroduplex analyses were performed using automated HPLC instrumentation with an Agilent 2G experimental dsDNA 2.1 x 75mm 3.5µ column (Agilent Technologies, Palo Alto, CA). Optimal melting temperatures for each fragment were estimated using an algorithm available at the Stanford DHPLC Melt program web page. Analytical acetonitrile gradients were composed by mixing Helix Buffer Pak A for DHPLC and 55-75% Buffer Pak B (Varian Analytical Instruments, Walnut Creek, CA) at a flow rate of 0.4 ml/minute. Samples displaying aberrant chromatograms were verified by direct sequencing.

3.2.3 Analysis of allelic imbalance (AI) (II)

The allelic imbalance at the *FH* locus at chromosome 1q43 was determined by analysing microsatellite markers. Three flanking microsatellite markers, D1S304, AL365184, and AL365366, were used. AL365366 (FH-T) was described earlier by Lehtonen and colleagues (2004) and primer sequences for AL365184 were 5'-CAT AAT TGG AAG CCA CTG GAG-3' for the forward and 5'-CAT CAC CCA ACT CAA GGT CA-3' for the reverse primer. PCR products were analysed using an ABI3730 DNA sequencer (Applera Corporation) according to the manufacturer's instructions and the data were analysed using GeneMapper 3.0 (Applera Corporation).

3.2.4 FH enzyme activity assay (II, IV)

An FH enzyme activity assay (Hatch 1978) was used to determine 1) whether the observed amino acid change has functional consequences (II) and 2) whether FH*v* functions as an active enzyme (IV). Wild type FH (FH_{WT}) was used as a positive control and non-transfected cells as a negative control. In addition, a mutated form of FH (FH_{H153R}), which is known to lack enzyme activity, was used as a negative internal control of transfected cells (unpublished data). The cDNAs of the wild type *FH* or *FHv* were cloned into pCI-neo Mammalian Expression Vector (Promega Corporation, Madison, US). The mutation K424R was created in an FH_{WT}/pCI-neo construct by site-directed mutagenesis (QuickChange® Site-Directed Mutagenesis Kit, Stratagene, La Jolla, CA) using primers 5'-CCT CAT ATA GGG TAT GAC AGG GCA GCA AAG ATT GC-3' and 5'-GCA ATC TTT GCT GCC CTG TCA TAC CCT ATA TGA GG-3' to generate the 1432A>G substitution. The constructs were verified by sequencing.

The wild type, variant, or mutated constructs (FH_{WT}/pCI-neo, FH*v*/pCI-neo, FH_{K424R}/pCI-neo, and FH_{H153R}/pCI-neo) were transfected into HEK293 cells (ATCC, Manassas, VA) using FuGENE 6 Transfection Reagent according to the manufacturer's instructions (Roche Applied Science, Indianapolis, IN). The levels of transfection efficiencies were confirmed by cotransfection of pEGFPN3 plasmid. Twenty-four hours after transfection cells were centrifuged into pellets and resuspended in 1 ml of 20 mM HEPES-KOH buffer, pH 7.5, containing 2 mM dithiothreitol. Suspensions were sonicated for 2 x 3 seconds on ice, centrifuged at 12,000 rpm for 3 minutes and the supernatants were collected. Cell lysates were stored at -70˚C. FH enzyme activities were measured as described earlier by Hatch (1978) with minor modifications: reactions were performed in a total volume of 260 µl and chicken liver malic enzyme (Sigma, Missouri, US) was used. The FH enzyme activity assay measures the amount of NADP consumed in the fumarate-to-malate reaction per minute per mg of total protein in the sample. Total protein levels of the cell lysates were measured using the BCA Protein Assay Kit (Pierce, Rockford, IL).

3.2.5 In silico analyses (II, IV)

To determine the influence of the observed amino acid substitutions on the protein structure (II), the SwissProt protein modelling server was used. In addition, the influence of the observed intronic polymorphism was predicted using the GeneSplicer Web Interface, Splice Site Prediction at the Berkley Drosophila Genome Project web page, and NetGene2 server.

To analyse the promoter and transcription binding site in *FH* exon 1b (IV), Genomatix tools Eldorado, GEMS Launcher, Gene2Promoter, and MatInspector were used. To investigate the conservation of the sequences, we used the ECR Browser (Ovcharenko et al., 2004), Ensembl database, and NCBI EST database (Boguski et al., 1993). The Webgene programme was used to make CpG island predictions. The 5' end of the *FHv* transcript was predicted using the CAGE (Cap-Analysis Gene Expression) database (Carninci et al., 2005), transcription start site database (Suzuki et al., 2004), or variant transcript databases (Alternative Splicing Gallery; Leipzig et al. 2004, Alternative Splicing Database Project; Clark and Thanaraj 2002, Thanaraj et al. 2004, Stamm et al. 2006). For internet addresses, see chapter 8.

3.2.6 Analysis of expression of *FHv* (IV)

3.2.6.1 Analysis of mRNA expression by quantitative RT-PCR

Quantitative real time RT-PCR was used to determine the mRNA expression of *FHv* in human tissues and cell lines. Total RNA from cell lines was extracted and transcribed into cDNA by M-MLV reverse transcriptase (Promega, Madison, WI). The cDNA from cell lines and tissue panels was amplified using cDNA specific primers and TaqMan chemistry (AB), and amplification was detected by the GeneAmp® 5700 Sequence Detection System (AB). All reactions were done in triplicates, and two human housekeeping genes, phosphoglyseratekinase and β-actin (*BA*), were used as endogenous control genes.

3.2.6.2 Analyses of expression and localization of FHv in cell lines

To study the expression and localization of *FHv*, the cDNA of *FHv* was cloned into pCI-neo Mammalian Expression Vector (Promega) and pEGFP-N3 vector (BD Biosciences Clontech). To determine the translation initiation codon of *FHv*, several constructs with different putative initiation codons were created in FH*v*-pCI-neo and FH*v*-pEGFP-N3 constructs using the QuickChange® Site-Directed Mutagenesis Kit (Stratagene). The constructs were verified by sequencing. The detailed structure of the constructs were described in the original article (IV).

Next, the constructs were transfected into HEK 293 cells using FuGENE6 transfection reagent (Roche, Applied Science, Indianapolis, IN), and the expression and localization were determined using a Leica TCS SP1 confocal microscope or an Axioplan upright epifluorescence microscope. Mitochondria were stained with MitoTracker (MitoTracker Red CMXRos, Invitrogen, Carlsbad, CA) and cells were fixed with 4% paraformaldehyde 24 hours after transfection. Nuclei were stained with Hoechst's nuclear stain and the endogenous FH was detected using porcine fumarase antibody (Nordic Immunology, Tilburg, the Netherlands) and FITC-conjugated goat anti-rabbit IgG.

3.2.6.3 Cell culturing and stress experiments

HEK293 and HeLa cells were cultured in DMEM, whereas HTB115 cells were cultured in MEM. The culture medium was supplemented with penicillin-streptomycin (100 U/ml and 0.1 mg/ml; Sigma-Aldrich) and with 5% FBS for HEK 293 and 10% FBS for HeLa and HTB115. The conditions were 37°C, 5% CO_2 and 21% O_2.

In stress treatment, cells were exposed to glucose or serum deprivation, hydrogen peroxide, heat shock, or hypoxia (details have been described in original article IV). After stress treatment, the total RNA and proteins were extracted. The mRNA expression levels of *FH* and *FHv* were measured with quantitative RT-PCR as described above (4.2.6.1). The protein levels were detected by Western blot.

3.2.6.4 Western blot analyses

The size and amount of either endogenous FH (stress experiment) or cloned FH_{WT} or FHv were determined by Western blotting. Total protein was extracted from transfected or stress treated cells, and 20-30 μg of protein was loaded into a 10% Tris-HCL gel. FH protein was detected using porcine fumarase antibody (1:500; Nordic immunology, Tilburg, The Netherlands).

4. RESULTS

4.1 The role of SDH in early onset renal cell carcinoma (I)

The search for early onset RCCs from the Finnish Cancer Registry revealed 244 unrelated cases. The expansion and documentation of pedigrees yielded 19 families with possible cancer backgrounds, of which one family with the most interesting family history was selected for further analyses (Fam1; Figure 4a). Fam1 included a 28-year-old index patient diagnosed with RCC. The histology of the tumour was clear cell carcinoma, showing a mixture of clear cells and cells with granular-eosinophilic cytoplasm. At the time of diagnosis, the tumour had already widely metastasized. The mother of the patient was diagnosed with PGL of the heart at the age of 55 years. The tumour was considered inoperable and therefore led to the death of the patient. In addition, two maternal uncles of the index patient were diagnosed with cancer, one with small-cell lung carcinoma at the age of 55 years and the other with pancreatic carcinoma at the age of 71 years.

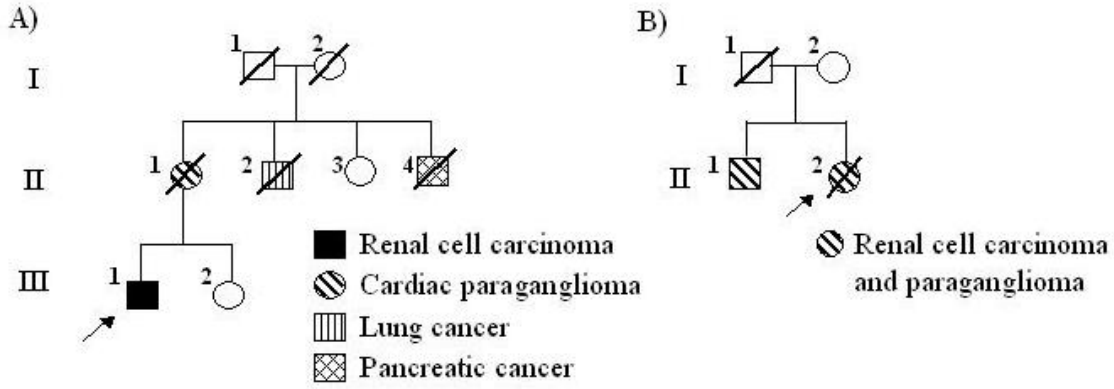

Figure 4. Pedigrees of the RCC families. The pedigree structures of Fam 1 (A) and Fam 2 (B) are presented. The probands of both families are indicated by arrows.

To determine whether *FH* mutations and HLRCC might explain the formation of these tumours, the *FH* gene was analysed by direct sequencing but no mutations were identified. Existence of the PGL of the heart in the family led to another hypothesis, as mutations in the FH preceding enzyme, SDH, have been reported in PGLs. To clarify whether SDH mutations could be the predisposing agent, all four genes encoding the subunits of SDH (*SDHA*, *SDHB*, *SDHC*, and *SDHD*) were analysed by sequencing. A truncating germline mutation R27X (129C>T) was identified in the *SDHB* gene. The index patient, his mother, and the uncle with lung carcinoma were all carriers of the mutated allele. LOH was observed in tumours of both the index patient and his mother, but not in the lung cancer of the uncle. DNA was not available from the other uncle with pancreatic carcinoma.

The other family included two siblings diagnosed with RCC of solid histology at the ages 24 and 26 years, and both patients had also suffered PGL (Fam2; figure 4b). Genomic DNA of the patients was analysed by direct sequencing and a germline frameshift deletion c.847-850delTCTC in *SDHB* was found in both patients. The loss of the wild type allele was observed in both RCC tumours and in the PGL of one patient.

Finally, *SDHA*, *SDHB*, *SDHC*, and *SDHD* were analysed in 95 sporadic RCCs to determine the frequency of SDH mutations in sporadic RCC cases, but no mutations were detected.

4.2 The role of *FH* mutations in tumourigenesis of early onset uterine leiomyosarcoma (II)

A total of 81 patients fulfilling the adjusted criteria for age were identified from the Finnish Cancer Registry. Paraffin embedded tumour tissues were available from 67 individuals. Altogether 52 out of 62 tumour-normal pairs available were informative for one or more microsatellite markers used to determine the AI at the *FH* locus. Nine tumours (17%) showed AI at the *FH* locus. In *FH* mutation analyses, a novel K424R (1342A>G) missense change was detected in one tumour. In addition, two silent polymorphisms were detected. One was in the coding region of exon 6 (927G>A) and the other in the coding region of exon 8 (1299C>T). Furthermore, 5 silent polymorphisms were observed in the flanking intronic sequences, but none of those were predicted to affect splicing *in silico*.

The patient with the K424R variant was diagnosed at the age of 41 years with a grade 2 ULMS of the uterine corpus. The disease had metastasized 10 years after diagnosis and the patient died 12 years after the original diagnosis. The patient had no other tumours and no tumours were reported in her first-degree relatives. The K424R variant was also found in the patient's normal tissue and no LOH or second hit were observed in the tumour tissue. The variant has not been published in dbSNP database. In addition, 280 healthy Finnish population controls were analysed for the K424R mutation by DHPLC to exclude the possibility of polymorphism but the change was not observed in the controls. Comparison of the protein sequence with other species showed that the amino acid K424 is highly conserved across higher species and *E.coli*, but *C.elegans* and *S.cerevisiae* do not share the lysine at the respective codon (Figure 5).

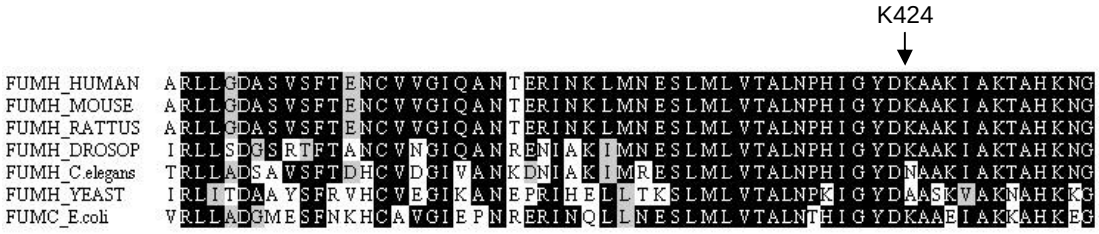

Figure 5. Sequence alignment of codons 377-436 of *FH* across species. *C.elegans* and *S.cerevisiae* do not share lysine at codon 424, which exists in higher species, human, *M. musculus*, *R. norvegicus*, and *D. melanogaster*.

The influence of the variant to the protein structure was predicted *in silico*, but no significant changes were observed. To determine the influence of the change to the function of the protein, an FH enzyme activity assay was performed in cell line model. The enzyme activity in FH_{K424R} transfected cells was 168 nmol/min/mg of protein, whereas in FH_{WT} transfected cells it was 390 nmol/min/mg of protein (Figure 6). Nontransfected cells and cells transfected with mutated FH (FH_{H153R}) with no enzyme activity (unpublished data) were used as controls. The activities in nontransfected cells and in FH_{H153R} transfected cells were 36 and 37 nmol/min/mg of protein, respectively. The levels of transfection efficiency were similar being 34% for FH_{WT}, 38% for

FH_{H153R}, and 42% for FH_{K424R}. Thus, the enzyme activity of FH_{K424R} was clearly reduced, being 43% of the wild type activity.

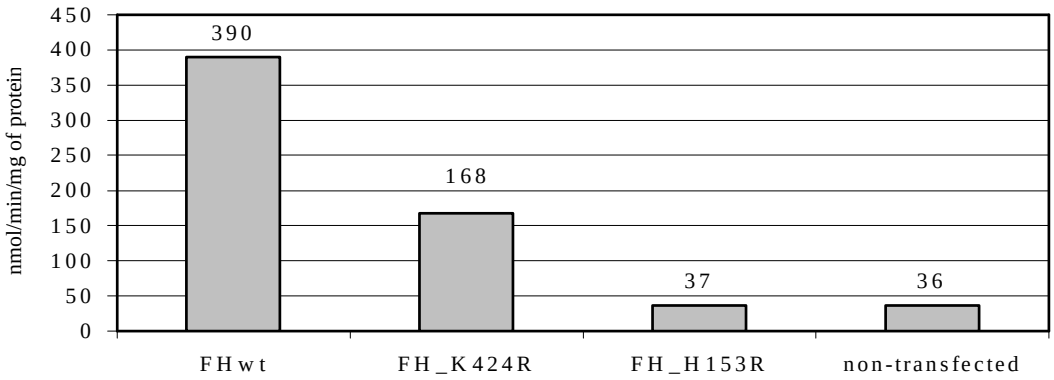

Figure 6. FH enzyme activity assay in a cell line model. The enzyme activity of FH_{K424R} was clearly reduced, being 43% of the activity of the wild type protein, FH_{WT}. Non-transfected cells and cells transfected with FH_{H153R}, which has no enzyme activity, were used as controls.

4.3 Specification of the spectrum of tumours associated with defective *FH* (III)

Altogether 150 samples from patients diagnosed with (1) RCC, skin leiomyoma, ovarian tumour, or (2) bladder carcinoma were analysed for *FH* mutations. First, blood samples of patients diagnosed with RCC, single skin leiomyoma or benign ovarian tumours were analysed in attempts to identify new germline mutation carrying HLRCC families. The analyses revealed two different *FH* variants in two patients, both diagnosed with ovarian mucinous cystadenoma (2/33, 6%). The first was a novel missense change A231T (691G>A), and the second a three base pair insertion (435insAAA). The patient with the A231T change was diagnosed with mucinous cystadenoma of the left ovary and with cysta endometrialis of the right ovary at the age of 25 years. She has no features or family background of HLRCC. Two second-degree relatives of the patient had been diagnosed with cancer, one with breast cancer and the other with leukemia. No tumour DNA from the patient was available for LOH studies. The 435insAAA insertion was observed in the DNA of the patient diagnosed with the mucinous cystadenoma of the ovary at the age of 49 years. She was also diagnosed with small multiple uterine myomas, but no other HLRCC features were observed. The father of this patient was also a carrier of the same alteration, but without a HLRCC phenotype. The mother of the patient carried normal *FH*. However, the mother and one first-degree relative of the patient were also diagnosed with ovarian tumours. No other familial background of cancer was observed in this family.

Because two germline variants observed in patients with mucinous cystadenoma are possibly connected to disease predisposition, an additional set of 13 mucinous cystadenocarcinomas were analysed to determine whether *FH* mutations also play a role in these malignant tumours. However, no mutations were detected.

Finally, 48 bladder carcinoma samples were included in the study. Bladder carcinomas had recently been diagnosed in some of the HLRCC families and this tumour type had

not been analysed for *FH* mutations earlier. However, no *FH* mutations were observed in the studied bladder carcinomas.

4.4 Characterization of *FHv* (IV)

A putative alternative form of *FH* was first identified from the AceView gene database. A 414 bp sequence (GenBank accession AI971779.1) containing exon 2, a part of exon 3, and an upstream sequence (exon 1b) was derived from ovarian tissue. The first exon of the mitochondrial *FH* encodes a mitochondrial signal peptide. The variant form of *FH*, *FHv*, contains an alternative first exon 1b which mapped to the intron between exons 1 and 2. The following exons (2-10) are equivalent in both forms (Figure 7). The exon 1b sequence was predicted *in silico* to contain no CpG islands, core promoter, conserved transcription factor binding sites, nuclear localization signals or hypoxia response elements. No evidence for the 5' extension of exon 1b was found in *in silico* studies, and the variant exon was not found in other species including *M. musculus, R. norvegicus, B. Taurus, C. familia, P. troglodytes* and *M. mulatta*.

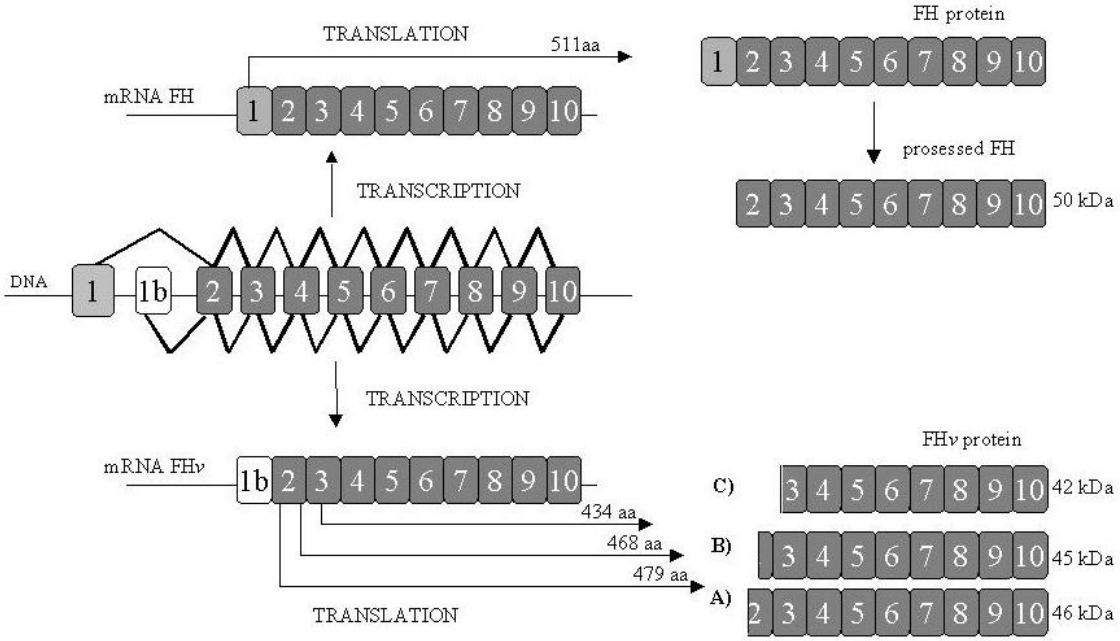

Figure 7. Structure of the *FH* gene. The organization of the exons and introns of both forms of *FH* (*FH* and *FHv*), and the structures of transcribed mRNAs and translated proteins are shown. According to our results, the translation of FH*v* is *in vitro* initiated from three alternative ATGs located in exons 2 and 3. Therefore, 3 protein products of different sizes are formed (A, B and C).

The mRNA expression of the variant *FHv* was measured by quantitative RT-PCR in human fetal and adult tissues and in cancer cell lines. The analyses revealed that *FHv* is expressed at low levels in all tissues. The expression of mitochondrial *FH* was approximately 200 fold greater in fetal tissues and 400 fold greater in adult tissues when compared to *FHv*. The *FHv* expression was highest in embryonic brain and in adult pancreas.

The expression of *FHv* mRNA was analysed by RT-PCR also in cell lines which were exposed to different stress conditions. Glucose or serum deprivation, H_2O_2 treatment or hypoxia (1% O_2) did not reveal any prominent changes in mRNA expression levels. However, the exposure of human uterine leiomyosarcoma cell line (HTB-115) to heat shock resulted in an increase in *FHv* mRNA expression levels: heat shock treatment for 30 minutes yielded a 3-fold increase in the expression levels, for 60 minutes 6-fold, and for 2 hours 9-fold increases, when compared to untreated cells **(**Figure 8a). A similar trend was also observed in HEK293 and HeLa cell lines (data not shown). Furthermore, in prolonged hypoxia (1% O_2, 72-96h), a tendency of increased *FHv* expression was observed. In hypoxia treated HTB-115 cells, the mRNA expression of *FHv* was increased 4-fold when compared to the untreated cells (Figure 8b). The same effect was also seen in HeLa and HEK293 cell lines. No increase in the expression of *FH* was detected under either condition (Figure 8). To confirm this finding, 12 additional cell lines were subsequently analysed. A trend of stress related induction was also detected in the half of these cell lines (6 out of 12; data not shown).

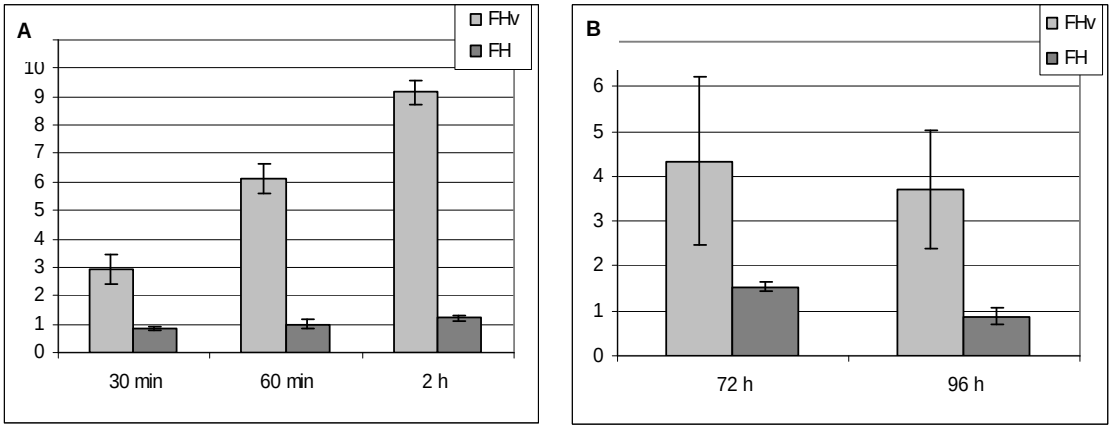

Figure 8. Relative mRNA expression levels of *FH* and *FHv* after heat shock and in hypoxia. A) The expression of *FHv* was increased 9-fold after heat shock treatment when compared to control cells. No increase was seen in the expression level of mitochondrial *FH* (FH). B) In prolonged hypoxia, the expression of *FHv* was increased 4-fold when compared to untreated cells. Again, no changes were seen in the expression levels of *FH*. Relative expression levels are calculated by comparing the expression levels of FH and FH*v* to their own expression in normal conditions and not to each other. Expression levels of untreated control cells were set to 1.0.

Variant exon 1b contains no ATG to act as an initiation codon. Instead, exon 1b contains two CTG codons which could function as possible initiation codons. In addition, there are three in-frame ATG codons in exons 2 and 3. The initiation of the translation of *FHv* was determined by transfecting HEK293 cells with FH*v*-GFP constructs containing different putative initiation codons. The putative initiation of translation in different constructs and the size of the translated protein was analysed by Western blotting. The Western blot analyses of the transfected HEK293 cells yielded three protein products (46 kDa, 45 kDa and 42 kDa), of which the middle one was very faint. The possible initiation of translation from either CTG was excluded by mutating the CTG to CCC, where all three bands remained in Western blots. When the CTGs were mutated to ATG, Western blot analyses revealed another larger band in addition to

those three bands. The same method of mutating ATGs to CCCs was used to further determine the correct initiation codon of the three ATGs in exons 2 and 3: when the first one was mutated to CCC, the largest band disappeared from the Western blot. Similarly, when the middle or the last ATG were mutated into CCC, the corresponding band vanished in Western blot analyses. Thus, translation was initiated from all three ATGs in exons 2 and 3, while the strongest expression occurred from the first and the last ATGs. Therefore, these results suggest that exon 1b is a 5' untranslated sequence of *FHv*.

The subcellular localization of the translated protein was determined using confocal immunofluorescence microscopy. Localization of the overexpressed FH*v* protein in transfected HEK293 cells was both nuclear and cytosolic, whereas the FH protein lacking both exon 1 or 1b was localized in cytoplasm. The endogenous FH or FH-GFP constructs were localized in mitochondria, while cytosolic fluorescence was also observed in a subset of FH-GFP overexpressing cells.

FH*v* was determined to have no typical FH enzyme activity. The activity in HEK293 cells transfected with *FHv* was 36 nmol/min/mg of protein, whereas in cells which were transfected with *FH* or mutant *FH* with no activity (FH_{H153R}, unpublished data), or were not transfected at all, the activities were 390, 35, and 36 nmol/min/mg of protein, respectively. Transfection followed by overexpression of *FH* caused a 10-fold increase in enzyme activity when compared to nontransfected cells. The activity in FH*v* or FH_{H153R} transfected cells and nontransfected cells were equal, indicating only the activity of endogenous FH.

In addition, the mutation status of *FHv* was determined in 118 patient samples and in 21 cell lines. However, this yielded no mutations in the exon 1b or its flanking sequences.

5. DISCUSSION

5.1 Mutations in the *SDHB* gene predispose to RCC (study I)

Early onset RCC has frequently been observed in Finnish HLRCC families (Kiuru and Launonen 2004). Therefore, we hypothesized that Finnish early onset RCC cases might include patients carrying *FH* mutations, and that examination of the familial cancer history could provide new HLRCC families. One family (Fam1) with a 28-year-old index patient was revealed from the Finnish Cancer Registry. *FH* mutation analysis of the index patient revealed no mutations, and therefore all four subunits of SDH (*SDHA*, *SDHB*, *SDHC*, and *SDHD*) were analysed. Although no mutations in SDH subunits had earlier been reported in RCC, SDH appeared to be a prospective candidate gene for several reasons. The mother of the index patient had been diagnosed with PGL and mutations in *SDHB*, *SDHC*, and *SDHD* have recently been reported in PGLs (Niemann and Muller 2000, Astuti et al. 2001, Carrel et al. 2002). A novel germline mutation, R27X, was identified in the *SDHB* gene. The index patient with RCC, his mother with PGL, and an uncle with lung cancer were carriers of this mutation. LOH was observed in both the RCC of the index patient and the PGL of his mother. No LOH was observed in the lung cancer of the uncle, and it is probable that the tumour had developed due to smoking over 40 years.

This is the first study in which *SDHB* mutations have been observed to predispose to RCC. However, even though LOH was observed in the tumour tissue, it can be argued that the occurrence of RCC in this patient carrying an *SDHB* mutation might be a coincidence and that this does not belong to the *SDHB*-paraganglioma syndrome. This suspicion was disproven by identification of another family (Fam2) with two siblings diagnosed with both early onset RCC (at the ages of 24 and 26 years) and PGL. Both patients carried a germline four bp deletion c.847-850delTCTC in the *SDHB* gene. Moreover, the RCC of both patients and the PGL of one patient showed LOH. These observations in two unrelated families confirmed that the existence of early onset RCC in these families is really caused by the observed *SDHB* mutations, and this phenotype did not occur by chance. Therefore, *SDHB* is a novel susceptibility gene for early onset RCC. However, RCC seems to be rare even among *SDHB* mutation positive families. In the PGL registry, only two RCC cases have been observed in one family among 16 *SDHB* mutation positive families including 31 mutation carriers. This led to a rough estimate that the approximate prevalence of RCC would be 5-10% among *SDHB* mutation carriers. However, patients included in the PGL registry are mainly young, with a mean age of 30 years (40% < 20 years, 15% > 40 years). Therefore, the true prevalence among *SDHB* mutation carriers can be somewhat higher, which should be taken into consideration in the clinical follow-up of patients with PGL and germline *SDHB* mutation. No mutations were observed in sporadic RCC cases and it appears that somatic *SDHB* mutations have a minor role in RCC.

Since 2001, numerous *SDHB* mutations have been reported in familial PGLs and PCCs, as well as in sporadic PCCs. However, no purely somatic mutations have been reported (van Nederveen et al. 2006). All types of mutations are reported in the *SDHB* gene and the mutations occur throughout the gene. No genotype-phenotype correlation has been observed (van Nederveen et al. 2006, Timmers et al. 2007). Interestingly, it has been assumed that *SDHB* mutations might be related to the malignant behaviour of tumours and that they generate a more aggressive type of PGL disease (Pawlu et al. 2005, van

Nederveen et al. 2006). Timmers et al. (2007) summarized that malignant disease has been observed in 55% of the cases, while the rates of malignancy varied between 34 and 97% in different studies.

Germline mutations in genes encoding two consecutive enzymes of the TCAC, *FH* and *SDH*, cause two different cancer syndromes with overlapping features. The early onset RCC seems to form a link between these two syndromes. Even though the histologies of renal tumours are different in these syndromes (mostly papillary type 2 in HLRCC and clear cell or solid histology in *SDHB* associated tumours), all these carcinomas originate from epithelial cells of the proximal renal tubule. Tumours of both syndromes seem to occur at unusually young ages, be unilateral, and especially aggressive. Both *SDHB* and *FH* seem to function as tumour suppressor genes and no mutations in either gene were detected in sporadic RCC. The consistency of these features indicates that at least some of the molecular mechanisms of kidney tumourigenesis associated with defective TCAC enzymes could be similar. Several hypotheses concerning these mechanisms have been proposed including e.g. mitochondrial dysfunction and apoptosis, ROS and oxidative stress, pseudo-hypoxia, inaccurate energy metabolism, activation of glycolysis, and unknown function of SDH or FH. Currently, the pseudo-hypoxia theory seems to be the one favoured by most researchers (for review see Eng et al. 2003 and King et al. 2006). Pseudo-hypoxia is also found in the background of the most common hereditary RCC syndrome, VHL (Latif et al. 1993). In addition to RCC, PGL also occurs in VHL patients. Mutations in *VHL* lead to stabilization of HIF1 and activation of hypoxia, glycolysis, and angiogenic pathways (Kim and Kaelin 2004). Therefore, it seems probable that a common factor underlying the tumourigenesis of RCC in all these syndromes could be the activation of the HIF1 pathway. However, this does not explain the occurrence of different tumour types (leiomyomas in HLRCC and PGGs/PCCs in patients with *SDH* or *VHL* mutations) in cancer syndromes caused by these genes. In addition, it appears probable that the tumourigenic effect of defective *SDH* or *FH* involves more than one mechanism (King et al. 2006). However, these mechanisms are probably not identical, since the majority of the features of these syndromes are different. The unifying tumour type, RCC, seems also to be relatively rare even among families with these syndromes, and the risk for RCC varies between families. Therefore, it has been proposed that a modifying factor together with a TCAC defect might be needed for the development of RCC (for a review see Eng et al. 2003). However, this modifying factor (or factors) as well as the molecular mechanisms behind TCAC associated tumourigenesis remain to be identified.

5.2 *FH* mutations predispose to ULMS and possibly also to ovarian tumours (studies II and III)

FH was determined to be a cancer predisposing gene quite recently. In studies II and III, we therefore attempted to clarify the spectrum of *FH* mutation associated tumours in more detail. Uterine leiomyosarcoma (ULMS) has been observed in Finnish HLRCC families and we hypothesized that *FH* mutations could play a role in the tumourigenesis of sporadic ULMS. HLRCC patients are diagnosed with ULMS at exceptionally young ages, varying from 27 to 39 years (Kiuru and Launonen, 2004), and therefore the criteria of early onset used in the Cancer Registry search was adjusted to ≤ 45 years (II).

FH mutation analysis revealed a putative novel germline missense mutation, K424R, in one patient diagnosed with a grade II tumour of the uterus corpus at the age of 41 years.

The variant was not reported in SNP databases and was not observed in 280 healthy population controls. In addition, we have analyzed several hundreds of Finnish samples for *FH* mutations in other studies and have never observed the K424R variant in those studies either. Therefore, it seems unlikely that the variant would be a common polymorphism. The amino acid K424 is strongly conserved across higher species but *in silico* protein prediction suddested no major changes to protein structure. The activity of FH_{K424R} was clearly reduced, being approximately 43% of the activity of wild type FH. However, FH_{K424R} still had some activity when compared to negative controls, but the reduced activity might not be enough for the normal function of the TCAC. These observations suggest that the variant could be a pathogenic mutation, although it is impossible to verify since no loss of the wild type allele was detected in tumour tissue and no segregation data was available from the family. In fact, several patients with HLRCC phenotypes and reduced FH enzyme activities but no detectable *FH* mutations have also been reported in earlier studies (Tomlinson et al. 2002, Alam et al. 2003, Pithukpakorn et al. 2006). Additionally, *FH* missense mutations have been observed to result in lower enzyme activities than truncating mutations, which is probably due to a dominant-negative effect: the FH protein functions as a homotetramer and a missense mutation in one allele results in only 1 out of 16 wild-type homotetramers (Tomlinson et al. 2002). It is also possible that the wild type allele has been silenced e.g. by hypermethylation at the promoter region, but it seems that the epigenetic inactivation of *FH* by hypermethylation at the promoter region is not common in leiomyosarcomas (Barker et al. 2006). Taken together, it appears that *FH* mutations may play a role in the pathogenesis of ULMS of young age at the population level. The frequency of *FH* mutations observed in these tumours is lower than expected based on the results from an earlier study in which Kiuru et al. (2002) detected one *FH* mutation in one ULMS out of 18 seemingly sporadic unselected cases (1/18, 5.6%). In the present study, one nonsyndromic ULMS with an *FH* mutation was detected in the population-based material (1/67, 1.5%). However, in both cases the mutation has been proven to be germline. These two studies suggest an estimate that the occurrence of *FH* mutations in Finnish non-syndromic ULMS is 2/85 (2.4%).

Uterine leiomyomas and ovarian cystadenomas without *BRCA1* mutations have recently been reported in Polish families with hereditary ovarian cancer (Menkiszak et al. 2004), and therefore ovarian cystadenomas were included in study III. Two different germline *FH* variants were found in two patients diagnosed with ovarian mucinous cystadenoma. The 25-year-old patient carrying the A231T missense change had no features of HLRCC and no familial background of HLRCC was observed. The lack of HLRCC phenotype might be due to the young age of the patient. The mean age at diagnosis of uterine leiomyomas in HLRCC patients is 31 years, and the relative risk at the age of 25 years has been estimated to be 9% (Alam et al. 2005c). The risk for skin leiomyoma at the age of 25 years is approximately 28% (Alam et al. 2005c). The 49-year-old patient carrying the 435AAA three bp insertion had been diagnosed with multiple small uterine myomas in addition to mucinous cystadenoma. No other features of HLRCC were observed in the patient or in her father, who was also a mutation carrier. This one amino acid in-frame insertion has been observed earlier in five FH deficiency families and no HLRCC features were reported either in homozygous patients or in heterozygous parents or siblings in these families (Gellera et al. 1994, Rustin et al. 1997, Coughlin et al. 1998, Loeffen et al. 2005, Deschauer et al. 2006). However, the insertion has never been detected as homozygous but always with a missense mutation in the other allele. In addition, no data concerning the function of the variant protein has been published.

Therefore, it is unclear whether this insertion predisposes to ovarian tumours. The *FH* mutation negative mother and one first-degree relative of the index patient were diagnosed with ovarian cancer and the predisposition for ovarian cancer can also be due to other, maternally inherited factors. Therefore, especially when no tumour tissue were available from either of these tumours and the LOH status remained unknown, it is impossible to conclude whether these variants are pathogenic mutations. Ovarian tumours have not been detected in HLRCC families and no *FH* mutations have been found from ovarian tumours in the earlier studies (Lehtonen et al. 2004, Lehtonen et al. 2006). Recently, several other benign tumour types, including atypical uterine leiomyomas, kidney cysts and adrenal gland adenomas, have been observed in *FH* mutation carriers (Lehtonen et al. 2006). Therefore, it appears possible that *FH* mutations might predispose to ovarian tumours, but more investigations are needed to verify these results.

Altogether, 142 sporadic tumours were analysed for *FH* mutations in studies II and III, and no tumours with purely somatic mutations were found. According to our results as well as results from earlier studies, it seems that somatic *FH* mutations are rarely enough for tumourigenesis. Thus far, numerous sporadic tumour types, including e.g. leiomyomas of uterus or skin, RCC, different sarcomas, breast and prostate carcinomas, and colorectal cancer, have been analysed for *FH* mutations (Lehtonen 2006). Lehtonen (2006) found purely somatic mutations in only three tumours (one in a soft tissue sarcoma and two others in uterine leiomyomas) out of 943 sporadic tumours analysed for *FH* mutations. Biallelic inactivation has been detected in almost all HLRCC tumours as well as in a few sporadic tumours with *FH* mutations (Lehtonen 2006).

5.3 Identification and characterization of a novel variant form of *FH* (study IV)

Localization and function of proteins are commonly regulated by removing interaction or localization domains. This can be done either by using alternative start sites for transcription or translation, or by alternative splicing (Mueller et al. 2004, Lareau et al. 2004). Recently, Schwerk and Schulze-Osthoff (2005) have also speculated that alternative splicing would have an important role in the regulation of apoptosis. A large number of apoptotic factors are regulated by alternative splicing and defects in those factors are also detected in cancer. It also seems plausible that alternative splicing is a mean to regulate intra-cellular messaging. Baek and Green (2005) have analysed a large set of human gene variants produced by alternative splicing and suggested that the key roles of splice variants are to regulate gene expression and mediate the destruction of intra-cellular messages. However, the large number of splice variants revealed to date also includes variants produced by aberrant splicing and it is challenging to distinguish these aberrantly spliced variants from functionally important ones (Baek and Green 2005).

Evidence of a putative novel variant form of *FH* mRNA was first published in the AceView gene database. Because *FH* is a novel cancer gene and its role in tumourigenesis was unclear, it was reasonable to study and characterize *FHv* in more detail. *FHv* mRNA was determined to contain an alternative first exon 1b, which, according to our studies, seems to be an untranslated 5' sequence. Two alternative forms of *FH* (*FH* and *FHv*) might be generated by different promoters, as described earlier for several genes with alternative first exons (Ayoubi and van de Ven, 1996; Hughes, 2006). *FHv* mRNA is expressed in all human fetal and adult tissues at low

levels. Interestingly, the expression levels of *FHv* increased significantly after heat shock treatment and in prolonged hypoxia. The induction was clearest in the HTB115 cell line but the same effect was also seen in approximately half of the other cell lines studied (8 out of 14; data not shown). Stress-induced expression of splice variants has also earlier been reported for several other genes such as *VEGF* and *COX1* (Turpin et al. 1999, Nurmi et al. 2005, Fay et al. 2006). Unfavorable growth conditions are known to result in heat shock response, in which heat shock factors induce the transcription and synthesis of heat shock proteins (HSPs), as well as several other proteins (Pirkkala et al. 2001, Sonna et al. 2002, Murray et al. 2004). HSPs are involved in the folding of proteins, regulation of the redox state, and in protein turnover. Under stress conditions, HSPs either promote the survival of the cell or induce apoptosis (Sonna et al. 2002). The increase in the expression level of *FHv* in unfavorable growth conditions might suggest that it plays a role in the adaptation of a cell to a disadvantageous environment or in stress related apoptosis. FH*v* could also be involved in the processing of cytoplasmic fumarate or in the shifting of energy metabolism from oxidative phosphorylation toward the glycolytic pathway. However, these hypotheses remain to be clarified.

FH enzyme activity was measured from both mitochondrial and whole cell extracts but no activity was observed. According to our results, translation of FH*v* is initiated from three ATGs located in exons two and three. This results in proteins in which the 5' end is 32, 43 or 77 amino acids shorter (initiated from the first, second or third ATG, respectively) than in the mature mitochondrial FH protein. This might have an effect on the formation of the homotetramer or on the catalytic activity of the protein and therefore result in the lack of enzyme activity. The significance of the absence of FH enzyme activity is unclear. If the function of FH*v* is unrelated to fumarate metabolism, the question of absent enzyme activity is irrelevant. It is also possible that *FHv* is produced by aberrant splicing and that it has no biological significance. Both of these hypotheses are possible and more studies are needed to disprove either of these alternatives.

FH*v* contains no mitochondrial signal peptide and localization of the FH*v*-GFP fusion protein was both nuclear and cytosolic *in vitro*, although no nuclear localization signals had been predicted with *in silico* analyses. Endogenous FH and FH-GFP protein localized in mitochondria, while a subset of FH-GFP overexpressing cells displayed also cytosolic expression. In yeast, the dual localization of FH has been described earlier and it appears that approximately 70% of fumarase is localized in cytoplasm (Sass et al. 2001, 2003). In mammalian tissues FH localized primarily in mitochondria but suggestive evidence of extra-mitochondrial localization of FH has been presented (Bowes et al. 2006). In addition, extra-mitochondrial localization has also been reported in several other primarily mitochondrial proteins (Mueller et al. 2004, Bowes et al. 2006). That FH*v* localization is not mitochondrial and that it seems to have no enzyme activity suggest that FH*v* might have some yet unknown function in the cell.

Taken together, we characterized a novel variant form of *FH* in more detail in this study. However, the role and the physiological significance of FH*v* remain unclear. The *FH* gene is highly conserved across species, but the counterpart of the variant exon 1b was not found in other species. FH*v* has no enzyme activity and although it is expressed in all human tissues, the expression levels are much lower when compared to the mitochondrial form of *FH*. *FHv* can be translated *in vitro* and it is not degraded

immediately, but endogenous expression of FH*v* has not been detected reliably at the protein level. Therefore, according to our results, it is evident that *FHv* mRNA is expressed in human tissues and the expression is induced in stress conditions, but the physiological function and significance of *FHv* remain to be verified.

6. CONCLUSIONS

HLRCC was characterized in 2001 for the first time and the predisposing gene, *FH*, was identified in 2002. In addition, mutations in another TCAC enzyme encoding gene, *SDHB*, were reported in PGLs for the first time in 2001. Because *FH* and *SDH* were novel cancer predisposing genes with well known but seemingly unrelated functions in the energy metabolism of cells, virtually nothing was known about their roles in tumourigenesis. Since 2001, increasing amounts of information concerning these genes and syndromes has accumulated, but there are still numerous open questions. This study aimed to answer some of these questions by defining the role of *FH* and *SDH* mutations in several tumour types and by characterizing a novel variant form of the *FH* gene.

I) The role of *FH* and *SDH* mutations in RCC were studied. Two novel *SDHB* mutations were detected in three RCC patients in two unrelated families. PGLs were also diagnosed in both families. This was the first study in which RCC was reported to be associated with *SDHB*-paraganglioma syndrome. However, RCC seems to be rare even among SDHB mutation carriers and no mutations were detected in sporadic RCC cases. Therefore, more studies are needed to confirm the prevalence of RCC in this syndrome.

II) The role of *FH* mutations were studied in population based material of early onset uterine leiomyosarcomas. One patient with a putative novel germline *FH* mutation (1/67, 1.5%) was detected in this study. The patient has no familial history of cancer and no LOH was observed in the tumour. However, the enzyme activity of the mutated protein was clearly reduced. Therefore, it seems that *FH* mutations play a minor role also in non-syndromic ULMS. One *FH* mutation has been detected in non-syndromic ULMS in an earlier study (Kiuru et al. 2002). Together these results suggest, as a rough estimate, that the prevalence of *FH* mutations in Finnish non-syndromic ULMS is 2/85 (2.4%).

III) This study aimed to identify new HLRCC families and define the spectrum of *FH* mutation associated tumours. Two different *FH* variants were detected in a novel tumour type, ovarian mucinous cystadenoma. Both variants were germline changes, but the patients had no familial history of HLRCC. In addition, no tumour tissues were available and the LOH status remained unclear. According to these results, it seems that at least certain *FH* changes might predispose to ovarian tumours. However, more studies are needed to confirm whether ovarian tumours should be included in the spectrum of *FH* mutation associated tumour types.

IV) In this study, a novel variant form of the *FH* gene was characterized, and the mRNA expression, translation initiation site and localization of this variant were determined. The expression of *FHv* seemed to be induced in unfavorable growth conditions which might suggest that it has a role e.g. in energy production or in the adaptation of the cell to disadvanteous environments. Currently, however, the physiological relevance of *FHv* remains obscure.

This thesis work defined the roles of two TCAC associated genes in several tumour types. Alterations in the *SDHB* and *FH* genes were observed in novel tumour types, which provide new information for both researchers and clinicians. In addition, the

characterization of *FHv* provided new information concerning this novel cancer predisposing gene. However, more investigations are needed to determine the additional functions of *FH* and *FHv* and to clarify the link between *FH* and tumourigenesis.

7. ACKNOWLEDGEMENTS

This study was carried out at the Department of Medical Genetics, Haartman Institute, and Biomedicum Helsinki, University of Helsinki, during 2002-2007. I would like to thank the former and present heads of the department of Medical Genetics, Anna-Elina Lehesjoki, Leena Palotie, Kristiina Aittomäki, and Päivi Peltomäki for providing excellent research facilities.

I thank the reviewers of my thesis, professor Anu Wartiovaara and docent Robert Winqvist, for their in-depth review and comments and ideas how to improve the thesis. Anu is also thanked for all other advice and help during this thesis project.

I have been privileged to work in an excellent research group and with two outstanding supervisors. I am grateful to Professor Lauri Aaltonen for giving me the opportunity to work in his lab and for his guidance and help during these years. Lauri is also thanked for creating a most inspiring working environment and an especial team spirit. I am also very grateful to my other supervisor docent Virpi Launonen for her excellent guidance, help, support, and encouragement whenever needed. You have been an admirable example how to work hard, get inspired time after time, and enjoy doing science.

I would like to thank the former and present members of the Aaltonen team; you have made this work not only possible but also enjoyable. My roommates Tuija, both Helis, Pia A, Taru, and Marianna are warmly thanked for their pleasant company and the discussions concerning the meaning of doing science as well as life outside of the lab. Tuija, already PhD, is specially thanked for sharing the whole pathway toward PhD beginning from the very first days in the lab when we both were novices in the world of the cancer genetics. Tuija is also thanked for the friendship outside the lab. I am also grateful to Heli L for her great contribution in the variant study. The former PhD students (nowadays PhDs) Maija, Susa, Päivi, Rainer, Antti, and Sakari are thanked for their help and company: Maija is thanked for her help in the beginning of my thesis project, Päivi for the riding company, Rainer for his help with all computer things and with dHPLC, and Sakari for his contribution in the SDHB study. Auli is warmly thanked for her guidance in the beginning of this project as well as for her help with Q-PCR and advice with the other practical issues. The sense of Auli's humour has made the long days in the lab much more fun. I am also sincerely grateful to Sini for numerous matters: For co-ordinating the data of samples, for taking care of the spirit of the group, for organizing the parties and recreation days, and for many other things – you are keeping this team going. I am also grateful for our excellent technicians, Inga-Lill and Mikko for their help during this project. Inga-Lill is specially thanked for excellent and extremely efficient way to work: You have always been so helpful even if you wouldn't have had time and your help during this thesis work is indispensable. I would also like to thank the other current members of the group, Anniina, both Iinas, Katja, Mairi, Mia, Pia V, Riikka, Sari, and Silva, as well as the past ones, Annika, Diego, Kirsi, Nina, Taija, and Ullis for their company.

I want to thank all my co-authors and collaborators: Johanna Arola, Mary Buchta, Ralf Butzow, Cezary Cybulski, Lars Dyrskjot, Charis Eng, Riitta Herva, Anu Jalanko, Andrzej Januszewicz, Matti Juhola, Heikki Järvinen, Jan Lubinski, Joanna Matyjasik, Sarah McWhinney, Jukka-Pekka Mecklin, Carl Morrison, Hartmut Neumann, Torben

Orntoft, Mariola Peczkowska, Eero Pukkala, Anna Szymanska, and Jolanta Szymanska-Pasternak.

Susanna Anjala, Heli Keränen and Pekka Ellonen are acknowledged for the excellent sequencing and fragment analysis services. I am also grateful to Jodie Painter for excellent and especially fast language revision of my thesis.

Heli, Iita, Maria, Jouni and Antti are thanked for many shared experiences as undergraduate students and after that. I wish to thank Anna, Johanna, Inga, Rami & Veera, Perttu & Emma, Miikka, Outi, Emilia & new baby, Pete, and Tiina, Jukka & Juho for showing that there is a true life outside the lab.

I am grateful to my parents Sinikka and Markku, my sisters Jaana and Tiina, and my grand mother Aune for their endless support and encouragement during this project: Without your help this work would have never been completed. Irmeli & Birger, Arto & Tiina, and Petri are also warmly thanked for being helpful whenever needed.

My deepest gratitude goes to my loving husband Tero, our wonderful child Eemil, and lovely dog Jaffa: You are always reminding me of the meaning of life and things that are really important. I am sincerely grateful to Tero for his endless support, love and care, as well as unselfish help during these years; thanks for everything!

This work was supported by Helsinki Biomedical Graduate School as well as by grants from The Research and Science Foundation of Farmos, Emil Aaltonen Foundation, and Ida Montin Foundation.

8. ELECTRONIC DATABASE INFORMATION

AceView Gene Database, http://ncbi.nih.gov/IEB/research/Acembly/index.html
Alternative Splicing Database Project, http://www.ebi.ac.uk/asd/
Alternative Splicing Gallery, http://statgen.ncsu.edu/asg/index.php
Berkley Drosophila Genome Project, http://www.fruitfly.org/cgi-pin/seq_tools/splice.pl
CAGE Basic Viewer for Homo sapiens, http://gerg01.gsc.riken.jp/cage/hg17prmtr/
DataBase of Transcriptional Start Sites, http://dbtss.hgc.jp
dbSNP, http://www.ncbi.nlm.nih.gov/projects/SNP/
ECR Browser, http://ercbrowser.dcode.org/
Ensembl Genome Browser, http://www.ensembl.org
Finnish Cancer Registry, http://www.cancerregistry.fi
Functional Annotation of Mouse-3 database (Fantom3), http://fantom3.gsc.riken.jp/
GeneSplicer Web Interface, http://www.tigr.org/tdp/GeneSplicer/gene_spl.html
Genomatix tools, www.genomatix.de
NCBI Expressed Sequence Tags database, http://ncbi.nlm.nih.gov/dbEST/index.html
NetGene2 server, http://www.cbs.dtu.dk/services/NetGene2/
OMIM, Online Mendelian Inheritance in Man,
http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=search&DB=omim
Primer3, http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi
Stanford DHPLC Melt program, http://insertion.stanford.edu/melt1.html
SwissProt protein modelling server, http://www.expasy.ch/swissmod/
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